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A BIOCHEMICAL, ULTRASTRUCTURAL AND
IMMUNOCYTOCHEMICAL ANALYSIS OF NUCLEAR MATRIX
IN CONCAVALIN A - STIMULATED LYMPHOCYTES

by

TREVOR SCOTT BLADON, B.Sc (Hons).

A thesis submitted to the Faculty of
Graduate Studies and Research in the partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

Department of Biology
Carleton University
Ottawa, Ontario

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ABSTRACT

Bovine lymphocytes, isolated and cultured with concanavalin A, were stimulated to enter G1 and S phases of the cell cycle. Nuclear volume increased greatly, condensed chromatin disaggregated and the interchromatinic space became enlarged and structurally complex.

Detergent-isolated nuclei retained *in situ* morphology and were used to isolate nuclear matrices (NM). NM from inactive and active cells had markedly different morphologies. Structural rearrangements occurred during extraction of chromatin from isolated nuclei and some nuclei shrunk during NM isolation. The latter phenomenon was not observed when NM remained embedded within residual cytoplasm, suggesting the cytomatrix and/or chromatin help to maintain an extended nuclear structure. The isolated NM is derived from nuclear lamina and interchromatinic material and could represent a nuclear skeletal framework.

Tight associations were observed between lymphocyte NM and 6-10nm cytoplasmic filaments. Nuclear matrices contained large amounts of vimentin and actin, indicating the filaments were intermediate or microfilaments.

Two-dimensional gels were essential for analysing complex nuclear matrix fractions. NM from unstimulated cells had simple composition of acidic to neutral proteins, consistent with their simple ultrastructure. Nuclear matrices from stimulated bovine lymphocytes had

complex polypeptide compositions, including numerous basic to neutral polypeptides. Increased compositional complexity arose from de novo protein synthesis and post-translational modifications. Nuclear matrix contains many transcription-associated proteins and may order RNA(P) metabolism.

Monoclonal antibodies were raised in mouse hybridomas against lymphocyte nuclear matrix. Immunofluorescence showed all antigens were more abundant in +conA lymphocytes. Antigens P1 (27k) and I1 labelled nuclear periphery and interior, respectively, were chromosome-associated in mitosis and were redistributed or removed from nuclei when chromatin was extracted. These highly conserved matrix antigens are probably important in chromatin organization throughout the cell cycle.

Antigens PI1 (60k) and PI2 (34k, 70k, 130k) labelled nuclear peripheries and interiors, were not redistributed during NM isolation and became cytoplasmic during mitosis. These proteins may be involved in RNA/P metabolism or binding components to NM. PI1 and PI2 are found within similar networks *in situ* and in NM, suggesting the NM is a real structure and does not arise from random precipitation of nuclear material.

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LIST OF ABBREVIATIONS

Ad	Adenovirus
8-AG	8-azaguanine
ALAT	Acyl coA:lysolecithin acyl transferase
BHK	Baby Hamster Kidney
CH	Cycloheximide
CHO	Chinese Hamster Ovary
Con A	Concanavalin A
DHT	Dihydroxytestosterone
DMEM	Dulbecco's Modified Eagle Medium
DNA	Deoxyribonucleic Acid
DNase	Deoxyribonuclease
DRB	5,6-Dichloro-1-B-D-ribofuranosylbenzimidazole
dsRNA	Double-stranded RNA
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EM	Electron microscope or electron microscopy
EtBr	Ethidium bromide
FITC	Fluorescein isothiocyanate
5-FU	5-fluorouracil
hnRNA	Heterogeneous nuclear RNA
HSB	High salt buffer
HSV	Herpes Simplex Virus
IA	Iodoacetate
IF	Intermediate filament
if	Interchromatin fibril

ig	Interchromatin granule
IL	Interleukin
IM	Interchromatinic material or matrix
kb(p)	Kilobase(pair)
LMB	Low Mg++ buffer
LPS	Lipopolysaccharide
2-ME	2-Mercaptoethanol
MF	Microfilament
MPF	Maturation promoting factor
mRNA	Messenger RNA
MT	Microtubule
MW	Mr - relative molecular mass
NaTT	Sodium tetrathionate
NB	Nuclear body
NE(G)	Nonequilibrium (gel)
NEPHGE	Nonequilibrium pH Gradient Electrophoresis
NHP	Nonhistone protein
NM	Nuclear matrix
NP-40	Nonidet P-40
NTPase	Nucleoside triphosphatase
PAGE	Polyacrylamide gel electrophoresis
PBL	Peripheral blood lymphocyte
PBS	Phosphate-buffered saline
PCL	Pore complex-lamina
pf	Perichromatin fibril
PHA	Phytohaemagglutinin
pHi	Intracellular pH

pI	Isoelectric point
PM	Plasma membrane
PMSF	Phenylmethylsulfonyl fluoride
pol	Polymerase
Poly(A)	Polyadenylate
ps	Post-stimulation
PWM	Pokeweed mitogen
rDNA	Ribosomal DNA
RNA/P	Ribonucleic acid/ribonucleoprotein
RNase	Ribonuclease
RNE	Residual nuclear envelope
Rnu	Residual nucleolus
rRNA	Ribosomal RNA
Sc1	Scaffold protein 1
SDS	Sodium dodecyl sulphate
SLE	Systemic Lupus Erythematosus
snRNA/P	Small nuclear RNA/ribonucleoprotein
STI	Soybean trypsin inhibitor
STM	Sucrose-Tris-Mg ⁺⁺ buffer
SV40	Simian Virus 40
Tag	Large T antigen
tRNA	Transfer RNA
TX-100	Triton X-100
UV	Ultraviolet
WGA	Wheat germ agglutinin

LITERATURE REVIEW

A. CHANGES IN MITOGENICALLY STIMULATED LYMPHOCYTES

1. General Physiological and Biochemical Responses

Lymphocytes comprise a major portion of the immune systems of vertebrates. Mature lymphocytes are small quiescent cells which can be stimulated to take part in immune functions. They can be isolated, relatively free of other cell types, from peripheral blood, spleen, lymph nodes or bone marrow. Lymphocytes are classified into two major groups based on function and embryonic origin. Bone marrow or bursa-derived "B" lymphocytes are involved in humoral immunity and can form immunoglobulin-secreting immunoblasts. The thymus-derived "T" lymphocytes take part in cellular immunity. This latter class of cells can be further subdivided based on function. "Helper" T cells can stimulate B or T cells, "suppressor" cells limit the immune response, and "killer" cells are involved in cytotoxic events (Beverley, 1977).

In order to provide their required physiological roles *in vivo* lymphocytes must respond to environmental stimuli. Lymphocytes isolated from blood, spleen or lymph nodes can be cultured *in vitro* for several days with no apparent changes. They remain a population of non-dividing cells in the G₀ or "resting" phase of the cell cycle (Setterfield et al, 1983). However, on addition of

an antigen a small proportion of the cells enters the G1 and S phases of the cell cycle, becoming lymphoblasts, with mitosis usually occurring 36-48 hours after incubation with antigen. This represents clonal stimulation. In order to elicit a more widespread activation *in vitro* it is possible to add non-antigenic mitogens to isolated cells. Plant lectins such as concanavalin A (con A), phytohemagglutinin (PHA) and pokeweed mitogen (PWM) are examples of such mitogens. Lipopolysaccharide (LPS) isolated from the outer membranes of Gram-negative bacteria also has mitogenic properties (Raetz et al, 1983). In contrast to antigens these substances stimulate a large proportion of the lymphocyte population to grow and divide, more or less non-specifically immunologically. Hence they are termed polyclonal mitogens.

The initial event in mitogenic stimulation of lymphocytes consists of mitogen binding to cell surface glycoproteins or "receptors" (Novogrodsky et al, 1979).

The saccharide moieties of the lymphocyte glycoproteins seem to provide specific binding sites for lectins at critical locations on the plasma membrane (PM).

Experiments have shown that a number of T cell mitogens with different saccharide specificities interact with physically-linked membrane sites. This indicates that many mitogens may react with the same receptor. However

it may also be true that different lectins interact with unique lymphocyte glycoproteins to induce blastogenesis (Novogrodsky *et al*, 1979). In addition, lectins preferentially bind to different cell types. For example, con A and PHA bind to and stimulate only T cells whereas LPS reacts only with B lymphocytes.

Recent experiments have aided in identification of at least one T lymphocyte receptor. T cells possess a number of cell-specific surface molecules (eg. T3, T11) identified by use of specific antisera. The T-cell antigen receptor (Ti) is a variably glycosylated protein, comprising a disulfide cross-linked heterodimer of molecular weight 90,000 (Chilson *et al*, 1984). Monoclonal antibodies against Ti linked to Sepharose are mitogenic.

Furthermore, Chilson *et al* (1984) have shown that PHA, con A, wheat germ agglutinin (WGA) and anti-Ti antibodies immunoprecipitate polypeptides with the same molecular weights (50,000 and 40,000) as found in the isolated Ti receptor. In addition, con A and PHA have greater

affinity for highly glycosylated forms of Ti. These results are consistent with the hypothesis that PHA and con A activate resting lymphocytes by binding to and cross-linking T cell antigen receptors. It has, however, not been proven that PHA stimulates growth via Ti, or exclusively via Ti, since PHA also binds to a variety of other cell surface molecules. Like the immunoglobulin

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molecules found on the PM of B lymphocytes, the T cell antigen receptor consists of polypeptides having both constant and variable regions. The result is a diversity of antigen receptors, each with a similar basic structure (Hood et al, 1985).

It is not clear whether the mitogens and their respective receptors must be internalized in order for cell activation to occur. Raffel and Sell (1982) found with rabbit peripheral blood lymphocytes (PBL) that endocytosis of con A, PHA and their receptors was within 30 minutes and that this process appeared to be coupled to stimulation. It was not clear whether activation occurred as a result of internalization or due to secondary effects from surface binding. When con A or PHA was immobilized on Sepharose beads or petri dishes to prevent endocytosis, lymphocyte proliferation was observed (Raffel and Sell, 1982). It is possible, however, that localized leaching of lectin from the surfaces occurred, followed by endocytosis.

Some workers have attempted to dissect the plasma membrane microenvironment around mitogen receptors in order to detect mitogen-induced changes. Resch and Rode (1979) enriched PM vesicle fractions from calf thymus lymphocytes for presumed con A receptors. In the same fraction they observed enrichment of (Na⁺, K⁺) ATPase and acyl coA: lysolecithin acyl transferase (ALAT). Only

these enzyme activities were found to be stimulated upon addition of con A. Enzymes uniformly distributed in the PM had suppressed activities with mitogen present. The enzyme ALAT transfers activated long-chain fatty acids into the phospholipid lecithin; it has particular affinity for polyunsaturated fatty acids. Thus con A addition causes greater membrane fluidity by altering lipid composition, leading to rapid diffusion of lipids in the PM.

As mentioned above, (Na^+, K^+) ATPase is also found near con A receptors and has increased activity upon mitogen binding. Several workers have studied lymphocyte cation fluxes before and after incubation with lectins. Kaplan (1977) reported that an important early post-stimulation (ps) membrane event was appearance of elevated Na^+ and K^+ fluxes after con A binding. The net effect of stimulation of the (Na^+, K^+) ATPase is increased Na^+ efflux and K^+ influx (Kaplan, 1979). Owens and Kaplan (1982) noted that these fluxes increase almost immediately in human cells. In murine lymphocytes incubated with lectins increased (Na^+, K^+) ATPase activity causes a sharp depolarization of the PM potential within two hours, followed by a recovery and hyperpolarization at 6 hours (Gerson and Kiefer, 1981). In addition, the intracellular pH (pHi) increases by 0.2 units 1-3 hours after the depolarization event. These changes are similar to those

observed in fertilized sea urchin eggs and in muscle cells activated by insulin. Cell activation is accompanied by altered membrane potential and pH in these systems also (Gerson and Kiefer, 1981).

If elevated cation fluxes are required for lymphocyte activation and/or maintenance of the activated state, then specific inhibition should prevent proliferation. Kaplan (1977) reported that addition of the ATPase inhibitor ouabain leads to loss of the proliferative response in human T cells. Hammarstrom and Smith (1979) found that addition of Rb⁺ ions, a specific inhibitor of the (Na⁺, K⁺) ATPase, blocked mitogenesis in mouse splenic lymphocytes stimulated by con A or LPS.

According to Kaplan (1979) the increased K⁺ flux after addition of con A to human PBL reaches a level twice that found in resting cells. If continued, this activity would quickly increase intracellular K⁺ concentration (K_i) to toxic levels. Two groups have recently studied outward K⁺ channels in PHA-stimulated human T lymphocytes. Using patch-clamp techniques, whole-cell K⁺ currents were measured. DeCoursey et al (1984) found that addition of PHA caused K⁺ channels to open during membrane depolarization, leading to outward currents of K⁺ twice the resting rate (Matteson and Deutsch, 1984); this compares with the increased (Na⁺, K⁺) ATPase inward current. Thus, K_i remains constant during lymphocyte

stimulation. The outward K^+ currents were inactivated as depolarization proceeded, due to voltage gating. Addition of K^+ channel blockers inhibits subsequent passage of the cells into S phase in a dose-dependent manner (DeCoursey et al, 1984).

Lectin stimulation of T lymphocytes causes an early Ca^{++} influx. Alternatively, the Ca^{++} ionophore A23187 alone has a mitogenic effect if added for 1-3 hours to mouse or human lymphocytes, when an additional signal is provided by 18 hours ps (Toyoshima et al, 1976; Hadden and Coffey, 1982). Removal of Ca^{++} from culture media in the first 20 h of lectin stimulation reversibly blocks entry of stimulated lymphocytes from G1 into S phase (Scott, 1985). Cadmium or manganese ions inhibit both Ca^{++} fluxes and mitogenesis in lectin-stimulated lymphocytes either by interfering with early mitogen-induced Ca^{++} influx or blockage of intracellular calcium triggering actions (Scott, 1985). Cd^{++} effects could include blockage of calmodulin or other calcium-binding proteins, interfering with a number of calcium-dependent processes which are essential for lymphocyte transformation (eg. see below).

In order for lymphocyte stimulation to proceed genetic expression must be altered in response to PM events. Presumably a "second messenger" is necessary to relay signals from the PM to the nucleus. Hadden et al

(1972) studied early changes in cyclic nucleotide metabolism in human lymphocytes stimulated by PHA or con A. They observed that cAMP does not stimulate lymphocytes in G₀ or early G₁ and noted further that agents which increase cAMP levels inhibit lymphocyte proliferation. Alternatively, within 20 minutes of mitogen addition levels of cGMP increase 17-fold (PHA) to 50-fold (con A) relative to resting cells. No increase in cAMP levels is detected.

Many mitogens (eg. con A, PHA, PWM, A23187) and at least one antigen are able to stimulate increased levels of cGMP and enhanced activity of lymphocyte guanylate cyclase. A specific inhibitor of guanylate cyclase inhibits the rise in cGMP levels in stimulated lymphocytes in direct proportion to its growth inhibitory effects. Inhibition is reversed by addition of exogenous cGMP (Hadden and Coffey, 1982). Since calcium is believed to regulate guanylate cyclase activity (Toyoshima et al, 1976) it is clear why early Ca⁺⁺ influxes are essential for lymphocyte activation. Furthermore it has been shown that Ca⁺⁺ and cGMP may be responsible for induction of early RNA synthesis in lymphocytes (Hadden and Coffey, 1982).

Lymphocytes are committed to proliferation by about 18-20 hours ps. Removal of con A from the PM by competition with alpha-methylmannoside prior to this period can prevent DNA synthesis measured at 48 hours ps

(Rudd et al, 1979; Toyoshima et al, 1976). Con A-induced mitogenesis requires a calcium-independent action at 15-18 h ps.

Cell-cell cooperation exists in the immune system, partially through production of soluble protein factors. Two such factors are IL-1 (interleukin-1, previously lymphocyte activating factor "LAF") and IL-2 (previously T cell growth factor "TCGF"). Both proteins increase T cell mitogenesis, support induction of cytotoxic T cell reactivity and aid in generation of helper T cells for antibody responses (see Gillis and Mizel, 1981).

Macrophages produce IL-1 which induces immature T cells to differentiate and produce IL-2. Interleukin-2 causes proliferation of effector T cells (clonal expansion). If murine B cells are cocultured with mitogen-stimulated T cells they are induced by T cell soluble factors (Owens and Kaplan, 1982).

The period of IL-2 production in culture occurs at 15-18 h ps. This corresponds to the time period when a calcium-independent action must occur to ensure commitment to DNA replication. It is believed that the second signal required for this commitment is an IL-2 induced increase in cGMP levels by about 20 h ps. This event is

accompanied by reduced cAMP concentrations, necessary for passage into the S phase (Hadden and Coffey, 1982). Thus it appears that lymphocyte proliferative responses are in

part differentially regulated by the antagonists cAMP/Mg++ and cGMP/Ca++.

An early and essential metabolic response observed after mitogen addition is elevated protein synthesis. Incorporation of radioactive amino acids into protein increases two-fold within the first hour ps (Grunert and Schafer, 1982) and rises more than 20-fold by 24h ps (Cooper and Braverman, 1977). The early increase in protein synthesis can be blocked within the first hour by cycloheximide, leading to a decreased proliferative response measured later (Varesio et al, 1980; see also, Setterfield et al, 1983).

The rate-limiting step in protein synthesis is not transcription. There is a 6 hour lag phase after addition of mitogen before increased messenger RNA (mRNA) synthesis is detected (Mitchell et al, 1978; Grunert and Schafer, 1982). In addition it has been shown that mRNA levels in lymphocytes always exceed the translation initiation rate (Cooper and Braverman, 1977).

The elevation in protein synthesis observed in stimulated lymphocytes is accompanied by increased processing and transport of preexisting nuclear RNA. Early in the activation process lymphocytes react primarily through changes in post-transcriptional pathways and not through alterations of the transcription program (ie. gene expression). The rate-limiting factor in

lymphocyte protein synthesis is the translational initiation rate, which is kept low by a number of factors.

Cooper and Braverman (1977) have studied ribosomal kinetics in resting and stimulated human peripheral blood lymphocytes. Over 70% of the ribosomes in resting lymphocytes are free 80S ribosomes not active in translation. This large pool of free ribosomes is maintained by continuous inactivation of newly synthesized ribosomal subunits and ribosomes completing translation. It is possible that the large pool of free ribosomes is maintained as preparation for the activation response. During the early stages of mitogenesis increased translation is, at least partially, due to activation of free ribosomes. Within two hours after addition of PHA to human PBL a drop in free ribosomes is detected, with concomitant rises in the numbers of native ribosomal subunits (active) and polysomes. Synthesis of new ribosomal RNA (rRNA) is not required for these changes. The process of ribosome activation continues for about 16h. Cooper and Braverman imply that ribosomes completing translation require a dissociation factor (DF) in order to dissociate into subunits capable of reinitiation. Failure to bind DF results in formation of a free 80S ribosome. It is suggested that resting lymphocytes have a shortage of DF and that cell stimulation leads to increased levels and/or activity of DF.

Resting lymphocytes are deficient in translation initiation factors. Addition of con A leads to elevated levels of these substances (Kay *et al.*, 1978). It has also been suggested that cytoplasmic inhibitors of translation are present in resting cells and that these lose activity upon con A addition (Kay *et al.*, 1978; Cooper and Braverman, 1981). Ahern *et al.* (1974) studied human and pig lymphocytes stimulated by PHA. Using a cell-free translation system containing a lysate of resting lymphocytes they found a deficiency in translation initiation. This could be overcome by addition of exogenous initiation factors, implying that PHA causes elevated levels of at least one initiation factor. Thomas *et al.* (1979) determined that the ribosome-bound factor eIF-3 required for initiation becomes active after mitogen addition. Cooper and Braverman (1981) suggested that phosphorylation of eIF-2 in resting cells may inhibit its action. In addition they found that the initiator-methionyl-tRNA (tRNA_i) is present at very low levels in unstimulated lymphocytes. Early after mitogen binding, there is an increase in tRNA_i levels such that a linear correlation exists between the translation rate and the amount of tRNA_i. Synthesis of all tRNA species examined increases during early stimulation such that tRNA_i and total tRNA levels are changed coordinately. These results are consistent with the hypothesis that

tRNA_i regulates the translation initiation rate.

A number of other changes in RNA metabolism occur early after mitogen addition. Resting lymphocytes contain about 0.17 pg RNA per cell and this value increases only slightly in the first 10 hours (Grunert and Schafer, 1982). By 40h the RNA content has increased several-fold, to 0.90 pg per cell. Ribosomal RNA (rRNA) synthesis increases almost immediately after addition of PHA while degradation of rRNA is markedly reduced. In resting cells almost one-half of rRNA is destroyed before use (Berger and Birkenmeier, 1979).

As noted above, initiation of mRNA synthesis only occurs 6h after addition of mitogen, i.e. long after protein synthesis has increased (Mitchell et al, 1978; Grunert and Schafer, 1982). During this period a number of RNA processing reactions are enhanced. For example, in bovine lymphocytes stimulated with con A, an increase is observed in the turnover rate of polyadenylate (polyA) tracts within the first few hours ps (Hauser et al, 1978). Grunert and Schafer (1982) detected a two-fold increase in polyadenylation of heterogeneous RNA (hnRNA or pre-mRNA) immediately after lectin addition. This results in a stabilization of these molecules and increased quantities of poly(A)-RNA in the nucleus. As this RNA is transported to the cytoplasm and is eventually translated, the net result may be a quantitative shift in gene expression

(Hauser *et al.*, 1978). Another RNA processing reaction activated upon con A addition is methylation of ribose and ribonucleotides. Within the first 60 minutes a steep increase in hnRNA methylation is observed, leading to much longer half-lives of the modified pre-mRNA species. Within the first 6h of lymphocyte activation the methylation rates of tRNA and rRNA also increase four-fold (Grunert and Schafer, 1982).

It has been suggested that lymphocytes are in a G₀ or "resting" state before mitogenic stimulation. From the discussion above it may be possible to conclude that activated cells enter G₁ phase of the cell cycle at about 16-20h ps when commitment to proliferation occurs. As will be discussed below, these first con A-activated lymphocytes enter into DNA synthesis (i.e. S₁ phase) at 28-30h ps, followed by mitosis before 48h ps (Setterfield *et al.*, 1983a). Other lymphocytes continue to undergo stimulation following the first group such that there is only poor synchrony of stages at any time. Analysis of populations for cell cycle kinetics or other parameters becomes complicated after this first wave of proliferation since any partial synchrony is lost.

2. Changes in Cytoplasm During Mitogenic Stimulation

Resting lymphocytes are a population of uniformly small cells with diameter of 4-6 μ m (Setterfield *et*

al, 1983a; Al-Hamdani et al, 1979). These cells have a thin rim of dense granular cytoplasm around the nucleus and very few organelles. Ribosomes are observed to be free, not clustered, in the cytoplasm. A few mitochondria and the centrosome are often positioned near a nuclear cleft and this area has been referred to as the "cell center". Emanating from the centrosome is a simple microtubule (MT) network, as detected by immunofluorescence (Rudd et al, 1979; Rogers et al, 1981) and electron microscopy (Schweitzer and Brown, 1984).

Upon mitogenic stimulation striking structural changes in lymphocytes are observed. Quantitatively the response appears the same regardless of cell source or whether the cells are stimulated *in vitro* (Setterfield et al, 1983a) or *in vivo* (Al-Hamdani et al, 1979; Setterfield et al, 1985). Lymphoblast formation is most clearly characterized by a nearly 6-fold increase in cell volume between 12 and 24h. The diameter of blast cells is usually greater than 10 μ m. Cytoplasmic volume is much greater in these cells than in resting lymphocytes and the cytoplasm is less (electron) dense. Clustered polysomes, rather than free ribosomes, are observed (Valkov and Moyne, 1974). In addition, mitochondria, rough endoplasmic reticulum and Golgi bodies are more common. In general the morphology of lymphoblasts suggests these cells have elevated levels of respiration and protein synthesis as

compared with resting lymphocytes (Setterfield *et al.*, 1983a, 1985).

The cytoskeleton of stimulated lymphocytes is far more extensive than that observed in resting cells. Modulations in the cytoskeleton occur during the first 10 minutes after mitogen addition (Rogers *et al.*, 1981). Con A receptors (and others) migrate to one pole of the cell, over the centrosome. This process is sensitive to cytochalasins but not to colchicine or taxol (Taylor *et al.*, 1971; Rogers *et al.*, 1981; Paatero and Brown, 1982), suggesting a microfilament (MF) but not a MT requirement. However MTs are believed to provide spatial control for the MF-dependent processes. This capping phenomenon also requires active cellular metabolism (Taylor *et al.*, 1971).

Stimulated lymphocytes exhibit more numerous cell surface microvilli, each containing a MF bundle (Loor, 1977; 1979). Transcription of the bovine lymphocyte B-actin gene increases almost 3-fold after con A stimulation (KecsKemethy and Schafer, 1981). The average actin concentration in mouse splenic lymphocytes increases two-fold within 24h ps (Waterhouse *et al.*, 1983).

Electron microscopic and immunofluorescent studies have shown a substantial increase in the MT network after con A activation of mouse splenic lymphocytes (Schweitzer and Brown, 1984; Waterhouse *et al.*, 1983; Rogers *et al.*, 1981). The number of MTs centered on the centrosome may be five

times greater in lymphoblasts. In addition there is a three-fold increase in average alpha- and beta-tubulin mRNA levels 40h after adding con A to bovine lymphocytes (Kecskemethy and Schafer, 1981). Peptide analysis of mouse lymphocytes has shown a population average 2.5-fold increase in tubulin content per cell, although due to asynchrony of the cell response this is likely an underestimate of the true increase in individual lymphoblasts (Waterhouse et al, 1983).

In summary, in order for the elaborations of the cytoplasmic MF and MT systems after lectin addition, increased transcription of the actin and tubulin genes is stimulated. Concomitant with these events is an increased MT assembly capacity of the centrosome (Schweitzer and Brown, 1984).

3. Changes in Nuclear Morphology and Function

The nucleus of the resting lymphocyte is nearly spherical and relatively small (3-4 μ m diameter). Nuclear pores are few but are usually prominent. The nucleolus is a small ribonucleoprotein (RNP) body with concentric fibrillar and granular zones (Tokuyasu et al, 1968; Valkov and Moyne, 1974; Setterfield et al, 1983a). A major proportion of the nucleus consists of large condensed chromatin clumps ("heterochromatin") subjacent to the nuclear envelope. Using Schiff-thalium stain for DNA it

is possible in the EM to resolve a thin rim of highly condensed chromatin between the lamina and peripheral condensed chromatin clumps (Valkov and Moyne, 1974). Condensed chromatin accounts for 50-60% of the total nuclear volume (Bladon, 1980; Setterfield et al, 1983a,b; Al-Hamdani et al, 1979). Between the condensed chromatin clumps lies a predominantly granular zone, termed the "interchromatin region", which stains positive for RNP (Bladon, 1980; Setterfield et al, 1983a) by the regressive uranyl EM stain of Bernhard (1969).

Historically this region has been referred to as "euchromatin", but the positive reaction to regressive uranyl acetate indicates that the major components are non-chromatin. The interchromatin region accounts for 40-50% of the nuclear volume in resting lymphocytes (Al-Hamdani et al, 1979; Setterfield et al, 1983a).

Transcription levels in these nuclei are very low and DNA synthesis is not detected by autoradiography.

Mitogenic stimulation of lymphocytes results in nuclear morphological changes in nuclei between 12-24h ps. Lymphoblast nuclei have approximately 4-8 times the volume of lymphocyte nuclei. The nucleolus becomes much larger (4.5-11.5-fold) and far more complex, with extensive fibrillar and granular regions and numerous lacunae (Valkov and Moyne, 1974; Setterfield et al, 1983a; Al-Hamdani et al, 1979). Use of regressive uranyl stain illustrates a

striking increase in the amount of interchromatin material. Morphometric analysis on EM micrographs shows that most of the increase in nuclear volume is due to a 7.5- to 10-fold expansion of this zone (Gladon, 1980; Setterfield *et al*, 1983). More obvious in lymphoblast nuclei are the 15-25 nm granules, 3-10 nm fibrils and amorphous material found here (Setterfield *et al*, 1983). Condensed chromatin masses become smaller and far more numerous in blasts, while heterochromatin volume increases only 1.5- to 2-fold (Setterfield *et al*, 1983a; Al-Hamdani *et al*, 1979). Many of the clumps are located in the nuclear interior and are not near the nuclear envelope. Thus it would appear that most condensed chromatin becomes disaggregated into smaller clumps but not fully decondensed to euchromatin. This change is easily observed after EM staining of DNA by Schiff-thallium. In addition, in guinea pig lymphocytes stimulated with PHA a thin rim of condensed chromatin remains at the nuclear membrane between nuclear pores (Valkov and Moyne, 1974). The nuclear structural changes observed during lymphocyte stimulation have been correlated with increased transcriptional activity. Interestingly the opposite sequence of changes occurs in the nuclei of maturing avian erythrocytes, during nuclear deactivation (Dardick and Setterfield, 1976). Nuclear changes similar to those in stimulated lymphocytes are observed when avian

erythrocytes are reactivated in heterokaryons (Lafond et al, 1983).

Human and mouse lymphocyte nuclei from cells stimulated with con A have been subdivided into three arbitrary classes based on morphological appearance. Type 1 nuclei resemble those in resting cells whereas type 3 nuclei are observed exclusively in fully-stimulated lymphoblasts. Type 2 nuclei are intermediate between these extremes (Setterfield et al, 1983a,b). These morphotypes are easily identified in light microscope sections and aid in assaying structural and biochemical changes which occur in cell populations undergoing blastogenesis asynchronously.

By microspectrophotometry of isolated mouse lymphocyte nuclei stained for protein Hall (1982) was able to reach several conclusions. Con A stimulation results in a 1.6-fold increase in total nuclear protein content and an average 1.8-fold increase in nuclear nonhistone protein (NHP) content within the first 24h ps. Although type 3 nuclei may contain as much as six times the NHP as type 1 nuclei an average increase of 2.8-fold is observed. NHP content is proportional to nuclear volume and the major increase in nuclear NHP occurs between 10-18h ps, corresponding to the period of nuclear morphological change.

The protein synthesis inhibitor cycloheximide (CH)

can block structural changes in nuclei of mouse or human cells if added up to 24h ps, although the greatest effects are noted before 20h (Setterfield et al, 1983a). This indicates that some protein(s) required for transition from lymphocyte to lymphoblast nuclei must be synthesized late in the activation sequence, during the period of actual structural changes. Scintillation counting and autoradiography indicate that proteins synthesized between 16-24h ps are relatively stable molecules; i.e. they turn over fairly slowly (Hall, 1982). Autoradiography of human lymphocytes labelled with [3H]-leucine shows greatest label over large stimulated nuclei, with decreasing amounts over smaller nuclei. Most (80%) of the stable nuclear protein after a 24h chase lies over the interchromatinic region (Setterfield et al, 1983). These changes in nuclear morphology are stable. Addition of CH or the transcription inhibitor amanitin between 32-40h ps causes no change in morphology of fully enlarged lymphoblast nuclei, suggesting that continued synthesis of protein or RNA is not necessary to maintain altered nuclear structure (Setterfield et al, 1983a).

The nuclear morphological and composition changes discussed above precede DNA replication. Replication of DNA initiates in the first cells more than 28h ps, with increasing numbers of cells recruited to at least 72h ps. Autoradiography of human cells fed [3H]-thymidine shows

that at 24h ps many type 3 nuclei are present but none are labelled, while at 36h ps some type 3 nuclei are labelled. High resolution autoradiography of 5 minute [3H]-thymidine pulsed cells indicates that most (85%) DNA synthesis occurs in dispersed and partially dispersed chromatin ("euchromatin") with minor amounts of label (15%) found over aggregates of condensed chromatin (Setterfield *et al.*, 1983). Mitoses are observed beginning at 44-48h ps.

Recent experiments have shown that a very early nuclear event after addition of con A to lymphocytes is enhanced DNA repair activity. In mouse or human cells stimulated with con A six-hour pulses of purine and pyrimidine analogues allow normal blastogenesis but prevent DNA replication measured at 48h ps. Regardless of pulse time (0, 12 or 24h ps) 8-azaguanine (8-AG) or 5-fluorouracil (5-FU) irreversibly inhibit DNA synthesis, although cells can pass from G₀ into G₁ (Boumah *et al.*, 1984). Greer and Kaplan (1983) have determined that resting human and mouse lymphocytes normally have about 3,200 DNA strand breaks per diploid genome. These lesions are repaired within the first two hours of con A stimulation. The analogues 8-AG and 5-FU apparently increase the number of lesions in DNA and block the break-repair process. The effect is to inhibit subsequent initiation of DNA replication. This observation may explain the extreme susceptibility of resting lymphocytes

to radiation damage (Johnstone and Williams, 1983). DNA strand breakage may be a general differentiation mechanism which limits cell proliferation. For example, as chick embryo myoblasts fuse to form myotubes during differentiation, single-stranded breaks appear in nuclear DNA (Johnstone and Williams, 1983). Thus lesions in DNA may act to hold resting lymphocytes part-way through a differentiation process. In addition, these breaks may allow extreme chromatin folding and compaction in resting lymphocyte nuclei. A stimulatory signal (eg. lectin, antigen) may induce rapid repair of DNA strand breaks, allowing continued differentiation and condensed chromatin disaggregation (Greer and Kaplan, 1983). In this regard it is tempting to speculate that the numerous lymphoblasts which fail to initiate DNA synthesis *in vitro* may be deficient in this repair activity.

Johnson et al. (1974) studied early changes in nuclear NHP after con. A stimulation of horse PBL. An early increase in phosphorylation of acidic NHP was detected as early as 2h ps with a 10-fold increase measured at 8h ps. The major species phosphorylated had molecular weights of 28,000 (28k), 52k and 90-130k daltons. Phosphorylation of acidic NHP has also been studied in mouse lymphocytes stimulated with LPS (Stetk and Williamson, 1978b).

Increased phosphorylation is detected in all polypeptide peaks in electrophoresis, with 4-9-fold greater

phosphorylation before 8h ps. A polypeptide at 115k becomes highly phosphorylated, up to 17-fold, by 68h ps. It appears that maximal phosphorylation of nonhistones occurs before substantial *de novo* synthesis of protein after stimulation. Thus it is likely that pre-existing molecules are post-translationally modified after LPS addition.

Another early event observed by Johnson et al (1974) was that, within minutes of adding con A, presynthesized [3H]-leucine labelled cytoplasmic NHP apparently enter the nucleus and bind tightly to "chromatin". Stott (1982) found that in the first 4h after adding LPS to mouse lymphocytes certain NHP appear in the nucleus after cytoplasmic synthesis and transport. It is not clear however, whether *de novo* protein synthesis is required. Histone migration to the nucleus is insignificant or absent during the same period. With human PBL Polet (1983) found that in the first 8h ps there was no detectable increase in NHP content per nucleus but a constant increase in nonhistone occurs between 12-24h ps. This result confirms the data of Hall (1982) with mouse lymphocytes. A parallel event noted in these cells is an increased uptake of [3H]-actinomycin D by lymphocyte chromatin. Polet (1983) inferred from this result that the newly synthesized NHP were partially responsible for changing the chromatin conformation. The rapid changes

occurring in NHP content, nuclear structure and condensed chromatin conformation are nearly simultaneous.

Another event which parallels the increased nuclear NHP content is a general decrease in cellular protein breakdown. In human cells stimulated with con A Polet (1983;1985) found that other compounds which inhibit proteolysis (eg. fluoride) also cause increased NHP transport to the nucleus and elevated [3H]-actinomycin D binding. Since these agents are not mitogenic it is likely that changes in nonhistone protein metabolism do not cause blastogenesis but are effects of the mitogenic process. It is believed that con A and other agents inhibit proteolysis, and increase NHP transport, via the lysosomal pathway (Polet, 1983;1985).

Stott (1982) studied [35S]-methionine labelled nuclear NHP from LPS-stimulated murine lymphocytes using two-dimensional electrophoresis. At 8h ps synthesis of a few polypeptides was stimulated 12-35 fold, although most species were synthesized at rates 2- to 7-fold the resting cell rate. By 24h ps the intensities of numerous spots on the gels were much greater and a few new species had appeared. The degree of stimulation of synthesis and the time of maximum stimulation varied for different proteins. Some NHP spots showed little or no change by 24h ps, whereas others showed a several-fold increase in intensity (Stott, 1980;1982). Presumably the latter class of

molecules had essential structural and/or functional roles in the large lymphoblast nuclei.

B. THE NUCLEAR MATRIX

1. Characterization of the Nuclear Matrix

The eukaryotic nucleus is an extremely complex structure, in terms of both structural elements and functions. Numerous attempts to ascribe specific functions to particular nuclear structures have been made. The first great success in cell biology was the merging of genetics and cytology in the demonstration that chromosomes (i.e. chromatin) represent precise linear arrays of genes. The nucleolus is established as the site of ribosomal RNA (rRNA) gene transcription and processing. A number of other nuclear functions exist but it has been only recently that structure-function relationships have been proposed. These include DNA replication and repair, transcription, RNA processing and transport and nucleocytoplasmic exchange. They will be discussed below in the context of their relationship to a proposed nuclear skeletal framework.

The structural components of nuclei have been described by many and in general the *in situ* nucleus can be subdivided arbitrarily into four areas:

- (1) The nuclear periphery consisting of a

double nuclear membrane with associated pores. A dense lamina 20-100nm thick exists beneath the inner nuclear membrane.

(2) The nucleolus consisting of fibrillar and granular zones, enclosing lacunae and 'vacuoles', respectively.

(3) Condensed or heterochromatin often seen next to the inner membrane but also found in aggregates or clumps in nuclear interior.

(4) Interchromatin material (or nucleoplasm) containing dispersed "euchromatin", gene transcripts and other material. The interchromatin granules (ig), perichromatin granules (pg), interchromatin fibrils (if) and coiled bodies found here were described by Monneron and Bernhard (1969) using the regressive uranyl RNP stain of Bernhard (1969).

In order to determine the chemical nature of nuclear structural element(s) nuclear constituents have been removed chemically or enzymically. In early studies acidic buffers were used to extract basic histones from nuclei, leaving a residual structure. Electron microscopy of residual interphase nuclei, after DNase digestion and histone extraction showed intact sponge-like residual structures (Georgiev and Chentsov, 1960). Later studies by Smetana et al (1963) and Narayan et al (1967) indicated

these structures might be RNP networks.

Berezney and Coffey (1974,1977) attempted to remove as much material as possible from isolated rat liver nuclei without destroying their spherical integrity. Nuclei were extracted using low and high ionic strength (2M NaCl) buffers, nonionic detergent and high concentrations of RNase A and DNase 1. These treatments removed 90% of nuclear protein, 99.9% of DNA and 98% of RNA and phospholipids. Protein yields have subsequently varied between 10% and 40% of total nuclear protein (eg. Berezney,1979; Fisher et al,1982) depending on methodology and inhibition of endogenous proteases. Berezney and Coffey (1974) termed the residual nuclear structure the "nuclear protein matrix".

EM observation of thin sections (eg. Berezney and Coffey,1977; Comings and Okada,1976) or whole mounts (Comings and Okada,1976) of liver nuclear matrices showed a structure very similar to the *in situ* interchromatinic network described by Monneron and Bernhard (1969). Three major zones were identified in the isolated nuclear matrix; namely, a residual nuclear envelope (RNE), residual nucleolus (RNU) and intranuclear matrix (IM). The RNE was a continuous electron-dense layer 10-15nm thick containing nuclear pore complexes. The interpore material was predominantly fibrillar when viewed in tangential section or in whole mounts. The RNU contained

identifiable fibrillar and granular zones and vacuoles. The IM resembled the *in situ* interchromatin space, consisting of a dense network of interconnected 3-5nm fibrils and 15-25nm granules, continuous with the RNE. Large electron-transparent areas within the IM were presumed to correspond to sites where condensed chromatin had been located in isolated nuclei (see also Long et al, 1979).

The 3-5nm fibrils of the IM of liver nuclear matrix were similar in size to fibrils observed *in situ* in if, pf, pg and nuclear bodies (NB). These fibrils appeared to coalesce into 7-12nm fibrils, forming a diffuse reticulum (Berezney, 1980b). Sonication of matrices, followed by differential ultracentrifugation, allowed isolation of these proteinaceous "matricin" fibrils. Polyacrylamide gel electrophoresis (PAGE) of this fraction showed three major species at 70k, 66k and 62k. This material might form the underlying skeleton in eukaryotic nuclei (Berezney, 1980a), permeating the entire intranuclear component (Comings and Okada, 1976).

Nuclear matrices with similar morphology and composition have been isolated from virtually every eukaryotic (non-plant) cell examined; for example, human (HeLa; Hodge et al, 1977), bovine lymphocyte (Hakayasu and Ueda, 1981), mouse liver (Comings and Okada, 1976), avian erythrocytes (Lafond and Woodcock, 1983), amphibian oocyte

(Krohne et al, 1984), clam Maul et al, 1984), insect (*Drosophila*; Fisher et al, 1982), *Intrahymena* (Wunderlich and Herlan, 1977) and *Physarum* (Bekers et al, 1981). HeLa nuclear "scaffolds" prepared using polyanions instead of 2M NaCl were identical to nuclear matrices (Lebkowski and Laemmli, 1982b). EM studies of nuclear matrices from differentiated and undifferentiated Friend cells (Long et al, 1979), reactivated avian erythrocytes (Laford and Woodcock, 1983) and stimulated mouse lymphocytes (Setterfield et al, 1983a) have shown morphological alterations during cell stimulation. Nuclear matrices from "inactive" cells are relatively simple, with a sparse or nonexistent IM. On the other hand, matrices from metabolically "active" cells contain an elaborate IM resembling that in rat liver.

The polypeptide compositions of isolated nuclear matrix preparations have been analysed primarily by 1-D PAGE. These gels show a profile simpler than isolated nuclei, with very little (if any) histone and many nonhistone proteins (NHP) between 40-150k. Three major bands, referred to as "lamins", are usually observed between 60-70k (Berezney, 1984). Two-dimensional PAGE has shown that matrix polypeptides range from acidic (pI 5.0) to basic (pI 9.0) species. The profile of matrix proteins shown by this method is rather complex and poorly understood (Peters and Comings, 1980).

Comparison of matrix polypeptide profiles from different sources has shown that protein differences are present, depending on species, cell type, method of preparation and electrophoresis system. In general major matrix proteins seem to be largely conserved in mammalian nuclei. The single greatest variation between matrix preparations is the degree of cytoplasmic contamination. Light microscopy can show only massive cytoplasmic contamination; EM, which can reveal small, dispersed contamination is only rarely applied in this regard. Actin-containing microfilaments are usually disrupted by fixation and dehydration during EM sample preparation and are not observed in the microscope (Berezney, 1984). However actin is clearly resolved in 2-D PAGE (eg. Nakayasu and Ueda, 1983). Intermediate filaments (IF) are resistant to extraction during matrix preparation and may appear as a corona of filaments around nuclei (Capco et al, 1982). A tight association between IF and the nuclear matrix has been shown in cultured vertebrate cells but not in, for example, rat liver. In HeLa, cytokeratins and vimentin may account for 20-25% of matrix protein, compared to 10-15% for lamins. Cytokeratins (eg. 52k, 44k) and vimentin (57k) complicate analyses of matrix proteins, particularly in 1-D gels (Staufenbiel and Deppert, 1983).

To date there have been few comparisons of matrices

from inactive and active cells. Hodge *et al* (1977) detected no marked differences in polypeptides of HeLa matrix during the cell cycle. Similarly Berezney *et al* (1979a) did not observe differences between nuclear matrices from Zajdela ascites hepatoma and normal or regenerating rat liver. Kuzmina *et al* (1984) compared (by 1-D PAGE) nuclear matrices from normal and regenerating rat liver, log phase vs. lag phase CHO cells and two hepatomas. Matrices from the more active of these cells contained less of lamins A and C, more lamin B and more high MW polypeptides (150-200k).

Nakayasu and Ueda (1981, 1983) studied unstimulated bovine lymphocytes and observed major polypeptides of 43k (actin), 53k (vimentin) and 68k (lamin B). Up to 50 minor NHP were also resolved, together with some residual histone. Up to 1985 there has been no extensive comparison by 2-D PAGE of a single cell type during an activation sequence. In this regard preliminary comparisons of mouse lymphocyte and conA-stimulated lymphoblast nuclear matrices have been reported by Settenfield *et al* (1983b, 1985).

2. Nuclear Lamina

The nuclear envelope consists of a double membrane with proteinaceous pore complexes at points where the membranes meet. The nuclear lamina is a dense fibrous 20-200nm layer directly under the inner nuclear membrane. Although not visible in all EM preparations the lamina is apparently a ubiquitous component of eukaryotic nuclei. The lamina can be isolated from nuclear envelopes, in association with pore complexes, using a treatment combining high ionic strength buffers and non-ionic detergent (Aaronson and Blobel, 1975; Dwyer and Blobel, 1976). These buffers remove histones, DNA and 40-50k integral membrane proteins to yield the pore-complex lamina (PCL), which has a regular honeycomb-like appearance in the EM. SDS-PAGE of these preparations has shown that 40-50% of the residual protein is found in three bands between 60-70k. The remaining minor bands are observed between 30-200k (Dwyer and Blobel, 1976; Gerace et al, 1978).

The three major protein bands mentioned above have been named lamin A (70k), B (67k), and C (60k). Immunological characterization of these proteins has yielded information on lamina structure. Gerace et al (1978) raised antibodies against rat liver lamins in chickens and using immunofluorescence microscopy claimed that these stained antigens exclusively at the nuclear

periphery during interphase. A small amount of intranuclear fluorescence was also apparent in the micrographs. A number of different cultured vertebrate cells were stained with the antibodies with identical results. Later studies have confirmed these results in virtually every vertebrate cell type examined, suggesting a high degree of conservation. Ultrastructural localization in the EM by immunoperoxidase staining has shown a high concentration of lamins on the inner surface of the inner nuclear membrane with no other nuclear labelling.

Two-dimensional PAGE of the lamins resolves B at 67k (pI 5.7) and suggests similarity between A (69k) and C (62k) whose 3-4 isoelectric variants each are clustered between pI 6.8 and 7.2 (Kaufmann et al, 1983). When the separated lamin bands are digested for peptide mapping (lamins A and C have very similar, but not identical, maps regardless of the methods used; eg. trypsin (Shelton et al, 1980), cyanogen bromide or hydroxylamine (Lam and Kasper, 1979), V-8 protease (Gerace and Blobel, 1981)). In all cases the lamin B maps are quite different from those of A and C. These data led Shelton et al (1980) and others to suggest that lamin C may be a proteolytic fragment of lamin A. A number of experiments suggest that this is not true. *In vivo* pulse-chase labelling and *in vitro* translation of lamins suggest lamins A and C are

derived from different mRNAs (Gerace and Blobel, 1981). Laliberte et al (1984) isolated unique mRNAs for lamins A and C. After *in vitro* translation a 74k precursor of the 72k lamin A was detected. The 62k lamin C had a shorter mRNA.

Whereas mammalian cells have three lamins, avian erythrocytes have two, resembling A and B (Stick and Krohne, 1982), and amphibian and clam oocytes have one (67-68k; Krohne et al, 1984; Maul et al, 1984). Xenopus erythrocytes have two lamins, both quite distinct from the cell-specific oocyte lamin (Krohne et al, 1984). The clam oocyte protein was similar to vertebrate lamins in terms of physical properties, modifications and epitopes. Maul et al (1984) raised antibodies against the 67k clam lamin. These recognized mammalian lamin B. Antibodies against rat liver lamins A and C cross-reacted with the 67k clam "lamin". These results suggest that one polypeptide may be able to perform all of the functions of the three mammalian lamins. The lack of chromatin binding to oocyte nuclear envelopes may preclude the requirement for lamins A and C in these cells. The use of antisera has shown that while epitopes are shared between different polypeptides the antigenic determinants of lamin "families" are complex.

When isolated nuclear envelopes are extracted with low and/or high ionic strength the association between the

lamina and the inner nuclear membrane remains intact. Treatment with 6M urea or 0.2N NaOH extracts lamins, suggesting they are peripheral membrane proteins. Lamin B is most resistant to extraction; 0.25M NaCl₂ extracts lamins A and C while a much higher concentration is required to remove some B. Gerace and Blobel (1981) suggested that lamin B may anchor the lamina to the nuclear membrane. Extraction of nuclear matrices or nuclear envelopes with 0.1M sodium carbonate (pH 11.5) solubilizes peripheral membrane proteins. Lebel and Raymond (1984) analysed by SDS-PAGE proteins so solubilized from rat liver nuclear fractions. Large amounts of all three lamins were found. The insoluble proteins included integral membrane proteins (eg. cytochrome p450) and a significant fraction of lamin B. The photoaffinity reagent azidopyrene labels integral membrane proteins, as well as lamin B. Lebel and Raymond (1984) concluded that the lamina was probably anchored to the inner nuclear membrane by lamin B, which is also at least in part an integral membrane protein.

The manner in which lamins form the dense lamina structure has received some attention. Lam and Kasper (1979) electrophoresed lamins and found monomers of A and C but homotrimers of B. Almost all of lamin B was detected in disulphide-bridged oligomers. Shelton et al (1980) found that lamins A and C also form homotypic

oligomers under mild oxidizing conditions *in vitro* and suggested this represented the native condition. The results of Shelton *et al.* (1982) indicated that lamin B was most often found in homotetramers, but was also observed in dimers and trimers. Lamin A was least likely to be observed in oligomers. Kaufmann *et al.* (1983) critically addressed the formation of lamin oligomers. When nuclear envelopes were isolated without 2-mercaptoethanol several disulphide cross-linked oligomers of lamins A and B were present. Lower MW non-lamins were not cross-linked, suggesting that lamins are arranged *in vivo* so these bonds could form. Nuclear envelopes isolated in the presence of disulphide blocking reagents contained no lamin oligomers. Kaufmann *et al.* (1983) indicate that sulphhydryl groups in lamins are clustered in hydrophobic domains. They have further suggested that the lamins are not cross-linked *in vivo* and that intermolecular disulphide cross links arise from *in vitro* manipulations. These cross links are apparently not required for stability of the nuclear envelope.

Immunofluorescence using antisera against rat liver lamins has shown that the lamina disappears during mitotic prophase. Most stain is dispersed over the cytoplasm. In late anaphase stain appears around the edges of chromosomal masses. Enhanced perichromosomal fluorescence is observed by telophase, coincident with reformation of

the nuclear envelope (Gerace and Blobel, 1978). During mitosis lamins A and C are soluble and can be immunoprecipitated from CHO cytoplasm. On the other hand, lamin B appears to be insoluble and membrane-associated during mitosis. Lamins labelled during the previous interphase are localized in G1 nuclear envelopes, suggesting that lamins are recycled (Gerace and Blobel, 1980). Two-dimensional PAGE of CHO proteins shows all three lamins to be more acidic during mitosis, due to up to 6-fold greater phosphorylation. This modification may be important in control of reversible lamina depolymerization during mitosis.

To study lamina disassembly during mitosis Jost and Johnson (1983) fused HeLa cells in mitosis and interphase (G1 or S) and stained the lamins. Fusing one or more mitotic cells with one interphase cell resulted in premature chromosome condensation after 15-30 minutes. Associated with this process was a complete dispersal of lamins to the cytoplasm by 40 minutes after fusion. Therefore, after a 15 minute lag, the interphase lamina was induced to depolymerize over a subsequent 25 minutes. As the ratio of mitotic to interphase cells increased the lag time was reduced. The authors concluded that a factor present in mitotic cytoplasm induced the formation of "mitotic" nuclei, with subsequent dissolution of the nuclear envelope. In a recent study Miake-Lye and

Kirschner (1985) induced early mitotic events in a cell-free system. Maturation promoting factor (MPF) isolated from *Xenopus* oocytes was added to thymocyte or CHO nuclei which had been incubated for 2 hours in *Xenopus* embryo cytoplasm. Within 25-30 minutes chromatin condensation was visible. Fifteen to twenty minutes later the nuclear envelope was completely dispersed. MPF addition caused increased phosphorylation of lamins A and C within 15 minutes, resulting in acidic shifts in pI for both proteins. Coincident with this was an abrupt weakening of the nuclear envelope (visualized as flattening), followed by a gradual loss of lamin staining. These results lend support to the model of Gerace and Blobel (1980) as discussed above.

Despite the fairly extensive characterization of the lamins few roles for the lamina have been firmly established. It seems plausible that this structure could organize and stabilize the nuclear envelope. Reformation of the envelope during late mitosis could be directed by lamins. Lamin B resides in membranous vesicles during mitosis and appears around anaphase chromosome earlier than lamins A or C. Some workers believe that the lamins actively organize interphase chromatin by binding bases of DNA loops to the nuclear envelope (for review see Hancock and Hughes, 1982). Mouse DNA binds to isolated matrix proteins of 60-70k which have been dissociated by 2M NaCl

and 5M urea and attached to nitrocellulose (Comings and Wallack, 1978). Mullenders et al (1982) found that DNA-protein complexes from bovine liver cells contained up to 7 polypeptides of 57-72k. These proteins were not unequivocally identified as lamins. Lebkowski and Laemmli (1982b) found that lamins were the major DNA-binding proteins in histone-depleted HeLa nuclei. Metalloprotein interactions within these nuclei were important in maintaining interphase DNA in an ordered state. Less abundant proteins within metal-stabilized intranuclear networks were minor DNA-binding species. A difficulty arises when the lamina is inferred to play a direct role in chromatin organization since chromatin is found in replicon-sized loops throughout the cell cycle, not only in interphase. Minor intranuclear DNA-binding metalloproteins within interphase HeLa nuclei (Lebkowski and Laemmli (1982b) are enriched in mitotic chromosome scaffolds and appear to be important in DNA-binding and chromosome condensation, after dissolution of the lamina (Lewis and Laemmli, 1982). These data suggest that intranuclear binding of DNA is important for long-term organization of chromosomes, while lamin-DNA interactions are transient in nature. In addition, the chromosome scaffold (see below) arises from intranuclear components in prophase and not the lamina (Bekers et al, 1981).

3. Reality of the Nuclear Matrix

Whereas the nuclear lamina is nearly universally accepted as being the salt- and detergent-resistant nuclear envelope (see above), the reality of the intranuclear component of nuclear matrices as a structural component of living nuclei has been questioned. While some authors (eg. Berezney, 1984) regard the intranuclear matrix network as a form of nuclear skeleton controlling nuclear structure and function, others (eg. Bouvier et al, 1985a,b) feel it could merely represent nucleoplasmic RNP (eg. transcriptional complexes) artifactually precipitated during matrix isolation. This controversy is impossible to resolve unequivocally at present.

A fibrogranular structure resembling the intranuclear matrix has been detected *in situ*. Ghosh et al (1978) observed HeLa and rat kangaroo cells with or without pretreatment with the transcriptional inhibitor α -amanitin. α -Amanitin clears chromatin and RNP from the interchromatin space, leaving a faintly stained fibrillar network. This protease- and RNase-sensitive network is not stained by Feulgen but can be stained by acidic protein stains. The interchromatinic matrix consisted of 5-6nm fibrils, sometimes associated with 15nm granules. In α -amanitin-treated chicken liver cells a homogeneous reticulate matrix of 10-15nm diameter fibrils, continuous with the chromatin and nuclear periphery, was observed by

EM (Brasch, 1982). The fibers were stained faintly using conventional EM or regressive uranyl RNP stain. A more intense staining pattern was observed after protein staining by phosphotungstic acid. Two days after administration of α -amanitin, nuclear morphology appeared normal, with the framework no longer visible. Brasch (1982) concluded that the nuclear matrix is a genuine component of nuclei which extends internally throughout nuclei from the nuclear periphery. Its appearance *in situ* is normally masked by associated nucleoproteins. The *in situ* matrix described by Brasch is structurally dissimilar to those isolated in solution, suggesting some rearrangement of components in the latter instance. It is possible that the fine fibrillar matrix observed within α -amanitin treated liver cells resulted from the drug treatment and did not exist in normal cells. However, inhibition of transcription in lymphocytes by DRB allowed resolution of similar networks within lymphoblast nuclei (Setterfield et al, 1985).

At least two lines of evidence suggest that the isolated intranuclear matrix may be an artifact. Kaufmann et al (1981) first reported that in rat liver the intranuclear matrix was only present after oxidation of protein sulphhydryl groups by, for example, sodium tetrathionate (NaTT). This compound was originally used as a protease inhibitor (Berezney, 1979). NaTT exerts its

effect by inducing formation of disulphide bonds between neighbouring protein sulphydryl groups. Adding iodoacetate (IA) to nuclei to block disulphide bond formation ultimately results in isolation of nuclear "ghosts". Kaufmann and Shaper (1984) concluded that disulphide bonds are not intrinsic but are formed during manipulations involved in matrix isolation. Addition of dithiothreitol (DTT) reverses disulphide formation and results in release of several NHP, including major non-lamins at 60-70k.

The results of Kaufmann *et al* (1981) have been disputed by numerous groups. For example, Van Venrooij *et al* (1982) isolated normal appearing matrices from HeLa nuclei in the presence of IA or 2-mercaptoethanol (2-ME). Abulafia *et al* (1984) studied the association between SV40 RNA and BSC nuclear matrix. When sulphydryl blocking reagents were added during matrix preparation no difference in the results was detected. The phenomena noted by Kaufmann *et al* (1981) may have been cell type-specific. Kaufmann and Shaper (1984) conceded that certain cell types may be more or less susceptible to disulphide formation. This indicates that other unknown phenomena may be involved in matrix stabilization.

Kaufmann *et al* (1981) also reordered the steps of nuclear extraction by pre-digesting nuclei with high concentrations of RNase. This led to subsequent

extraction of residual nucleoli and granular matrix components by high salt buffers. Bouvier *et al* (1985a) concluded that HeLa RNPs are responsible for stability of the high salt-resistant matrix. They suggested that RNPs interact to form an intranuclear structure bound to the lamina. Predigesting with RNase leads to release of several NHP, including hnRNP core proteins. Long and Schreier (1983) obtained similar results using Friend erythroleukemia cells depleted of chromatin using hypotonic solutions. It was found that hnRNA could be released by mild shear, trypsin or RNase, which also disrupted the intranuclear matrix. The presence of divalent cations and/or aggregation of RNase-sensitive fibrils could stabilize the matrix during salt extraction. Long and Schreier (1983) could not dismiss the possibility of a fragile in situ underlying matrix as suggested by Brasch (1982). Aggeler (1985) recently isolated a nuclear matrix from liver cells using a gentle procedure and this closely resembled the in situ matrix observed by Brasch (1982). RNA/P was believed to have no structural importance in this nuclear matrix. Several laboratories have questioned the role of RNA in stabilizing the nuclear matrix by isolating nuclear matrices after extensive RNase digestion. For example, Van Venrooij *et al* (1982) examined the RNA remaining in HeLa matrices and determined the molecules to be 1-50 nucleotides long. The authors

suggested it was unlikely that this RNA was important in the structural stability of the nuclear matrix.

As mentioned above, there have been suggestions that divalent cations may play roles in matrix structural integrity. For example, Lebkowski and Laemmli (1982a,b) showed that the intranuclear matrix is stabilized by metalloprotein interactions. Cu^{++} ions are thought to be responsible, with Ca^{++} ions less effective. Small et al (1985) reported that Mg^{++} ions were crucial for the integrity of matrix structure in high salt. Long and Ochs (1983), studying Friend cell chromatin-depleted nuclei, observed that addition of 2M MgCl_2 resulted in matrix fibril aggregation and reformation of the residual nucleolus. Brasch (1982) found that the conformation of the intranuclear matrix is dependent on the appropriate charge environment, such that high cation concentrations cause aggregation while hypotonic conditions result in matrix expansion. These results confirm the reversible contractility of isolated matrices from *Tetrahymena*, which expand and contract according to cation concentration and the molar ratio of $\text{Ca}^{++}/\text{Mg}^{++}$ (Wunderlich and Herlan, 1977).

The possibility that the matrix results from aggregation of proteins induced by high salt concentrations has been questioned on several occasions by groups using less than 2M NaCl . Van Eekelen and van Venrooij (1981) obtained matrices from HeLa cells using

0.25M ammonium sulphate. Polyanions in low ionic strength buffers have been used to extract nucleic acids from isolated nuclei, resulting in formation of intact nuclear matrices (Adolph, 1980). Long and Ochs (1983) isolated chromatin-depleted nuclei resembling matrices from Friend cells using hypotonic buffers and nucleases. Long et al. (1979) had earlier isolated nuclear matrices from these cells using isotonic solutions.

There have been suggestions that the pore complex-lamina (PCL) represents a subfraction of the nuclear matrix, rather than the internal matrix being artifactual. The intranuclear matrix is extremely protease-sensitive, requiring protease inhibitors and low temperatures for preservation of the fibrogranular network (Miller et al., 1978a; Berezney, 1979; Nakayasu and Ueda, 1981; Mariman et al., 1982). Neither of these precautions are routinely included in lamina isolations (eg. Dwyer and Blobel, 1976). The internal matrix is also very sensitive to shear (Long and Schrier, 1983; Berezney and Coffey, 1977). Comings and Okada (1976) observed intact matrices, lamina and broken matrices in the same preparation, leading to the same conclusion. The lamina preparations mentioned above included vortexing and centrifugation at high speed through low viscosity buffers.

As discussed at length below there are also numerous

functional differences between the pore-complex lamina and the nuclear matrix. These include DNA binding and replication, transcription and RNA processing, virogenesis and hormone receptor binding. In each case distinct differences are found with regard to interactions involving the lamina or the internal matrix.

At the moment, therefore I conclude that it is a reasonable working hypothesis that the structure and functions of eukaryotic nuclei are in part dependent on a skeletal structure comprised of a PCL and an internal fibrogranular network. These structures are isolated in variably modified form by a variety of nuclear matrix isolation procedures. Further experiments are crucial, however, in order to better characterize which factors are responsible for stabilizing both the *in situ* and isolated nuclear matrix. In addition, the components of the IM must be identified and characterized, with respect to their structure, chemical properties and precise *in situ* locations. Immunological probes against IM proteins may aid in regards to the latter investigations.

4. Chromosomal Scaffold

When isolated metaphase chromosomes are dehistonized and DNase- digested a residual NHP chromosomal scaffold is sometimes visible. Paulson and Laemmli (1977) extracted HeLa chromosomes using 2M NaCl or polyanions (dextran

sulphate or heparin) to remove histones and several NHP. The residual scaffold observed in the EM had the characteristic shape of a metaphase chromosome and was surrounded by a halo of 30-90kb DNA loops. These loops were anchored to the core at adjacent points.

Approximately 30 minor NHP were observed in the 8-10% of total chromosomal protein which remained (Adolph et al, 1977). When thin sections of intact swollen HeLa chromosomes were examined a central dense region was observed, surrounded by 10nm DNA fibers arranged in a radial loop fashion. These fibers were condensed into 20-30nm fibers and 50nm supercoils by histones when present (Marsden and Laemmli, 1979; Adolph, 1980a).

Expanding on these studies Earnshaw and Laemmli (1983) isolated chromosomes with far less cytoplasmic contamination (eg. actin and intermediate filaments) and observed a simplified scaffold composition; the scaffold proteins Sc1 (170k) and Sc2 (130k) accounted for about 40% of the scaffold protein mass. Whole-mount EM preparations of these chromosomes in low ionic strength buffer resembled "puddles" of chromatin, with no paired chromatid morphology and reduced chromatid "axis" visibility.

However in the presence of 5mM Mg^{++} chromatin contracted back onto chromatid axes. The two forms of residual scaffold, namely, diffuse (in EDTA) and condensed (in Mg^{++}) represent reversible alterations. Structurally the

scaffold contained residual kinetochores, axial elements and peripheral material. The structure was 95% protein and represented only 4-7% of total chromosomal protein. Marsden and Laemmli (1979) proposed that chromosomal structure can best be described by an axial scaffold which binds radial DNA loops at their bases. The scaffold represents a loose fibrous NHP network which extends throughout chromatids.

There are several similarities between the scaffold and the nuclear matrix. The former is a residual NHP network found throughout mitotic chromosomes while the latter is a NHP network found throughout interphase nuclei. Both structures result from dehistonization using elevated ionic strength or polyanions. In addition the sizes of DNA loops at metaphase and interphase are the same (30-100kb). Both types of structure are sensitive to cation concentration, reversibly expanding and contracting. This implies a constant order for chromatin throughout the cell cycle with differing domain organizations at each stage of the cycle. Okada and Comings (1979) found that when CHO metaphase chromosomes were extracted by 4M ammonium acetate, DNA rosettes (chromomeres) were formed. When interphase nuclei were treated with this salt, similar rosettes were observed in the EM. The authors could not discern whether NHP held the DNA loops together or whether they were

spontaneously-formed DNA structures. When Lewis and Laemmli (1982) treated metaphase chromosomes with metal chelators the scaffold NHP dissociated. Addition of Cu^{++} (or Ca^{++}) ions allowed regeneration of compact scaffold structure. Lebkowski and Laemmli (1982a,b) demonstrated similar metalloprotein interactions in interphase nuclear scaffolds. It remains to be shown that the same chelation-sensitive interactions in interphase are maintained throughout mitosis.

Possible relatedness between the interphase matrix and mitotic scaffolds would be reinforced by demonstration of shared polypeptides. Pieck et al (1985) compared bovine liver scaffolds and matrices by 1-D PAGE. The profiles from the two structures were similar but differences were observed. Lamins were major matrix proteins but were absent from scaffolds. Detke and Keller (1982) compared HeLa matrix and scaffold proteins by 1-D and 2-D PAGE. They confirmed the lamin observation and detected major scaffold-specific proteins at 35k, 70k and 130k. 2-D PAGE showed the matrix to consist primarily of acidic (pI 5.4-5.9) species between 40 and 70k. Chromosome scaffolds contained this group of polypeptides in addition to several others with pI 6.5-7.0 and MW 43-130k. Two major high MW species at pI 6.7-7.0 may have been Sc1 and Sc2.

Earnshaw et al (1985) have identified the major HeLa

scaffold protein Sc1 as DNA topoisomerase II. Antisera against both proteins cross-react in Western blots and give identical immunofluorescence of chromatid axes *in situ* or *in vitro*. Earnshaw et al (1985) conclude that DNA topoisomerase II represents a major structural component of mitotic chromosomes, which may be situated at the bases of DNA loops. Immunoelectron microscopy of Sc1 has shown no evidence for a rigid chromosomal scaffold but instead numerous separate "islets" of stain are observed in chromatid axes (Earnshaw and Heck, 1985). These islets are situated within clusters of chromatin loops which closely resemble the rosettes observed by Okada and Comings (1979). Topoisomerase interactions with DNA may result in formation of these clusters. Additional interactions between topoisomerase molecules may result in short range organization of scaffolds. Nuclear matrices from *Drosophila* and rat also contain Sc1/topoisomerase II. Immunofluorescent staining shows the antigen has an intranuclear localization *in situ* which is punctate and non-nucleolar. The stained spots may represent interphase clusters of chromatin, although further more detailed characterization is required to confirm this interpretation (Berrios et al, 1985).

Other evidence supporting a relationship between the nuclear matrix and chromosomal scaffolds has come from observation of thin sections or whole-mounts of nuclei

and chromosomes *in situ*. Bekers *et al* (1981) examined the nuclear matrix of *Physarum* during mitosis. In this system mitotic events occur within the nuclear envelope until late anaphase. The internal matrix is gradually transformed into a scaffold-like structure beginning in interphase. The authors suggest that the internal matrix may be responsible for folding chromatin fibers into mitotic chromosomes. Recent experiments have shown synaptonemal complexes are coisolated with matrices from pachytene nuclei of mouse spermatocytes (Ierardi *et al*, 1983) and flour moth oocytes (Raveh and Ben-Ze'ev, 1984). The SCs course throughout the intranuclear matrix and retain their fine structural organization after 2M NaCl extraction. The meiotic chromosome scaffold also appears to be derived from the intranuclear matrix. In early meiotic prophase of silkworm 20-30nm fibers form numerous loops and increasingly larger arrays. These coalesce to form foci within *in situ* nuclei. Adjacent foci appear to associate to form longitudinal axes of condensing chromosomes. Chromatin forms a series of radially arranged loops distributed evenly along the axes, similar to isolated mitotic scaffolds. At pachytene the paired chromosomes have a brush-like appearance (Rattner *et al*, 1980). The dynamic behaviour of scaffold proteins suggests they may be regulated in a cell-cycle dependent manner. A

mechanism of self-assembly under suitable conditions, as hypothesized for the lamins, would require fewer components and could represent a simplified system for chromatin modulation during the cell cycle.

The reality of the chromosome scaffold, like the nuclear matrix, has been questioned. Wunderli et al (1983) were unable to isolate scaffolds from cultured mouse cells and suggested that histones were essential for accurate chromosome organization. Labhart et al (1982) found no evidence of a shape-maintaining scaffold. Instead they concluded that all histones are required for maintenance of metaphase chromosome shape. These workers support a model whereby chromatin fibers are folded into higher orders of helical coiling. They conclude that the scaffold represents an artifactual aggregation of hydrophobic proteins. Okada and Comings (1980) reviewed other arguments against the scaffold and concluded the structures in whole mounts arose due to incomplete dispersion of highly concentrated chromatin at chromatid axes. Histone removal and DNA dispersion causes NHP aggregation according to the authors.

Possibly recognizing that many of the early chromosome scaffold preparations were largely artifact, Earnshaw and Laemmli (1983) addressed many of the above criticisms in their experiments. They isolated chromosomes by three different methods, dehistonized by

high salt or low ionic strength, illustrated potential artifact formation due to shear, sectioned intact chromosomes and showed that scaffold appearance is dependent on cationic environment. Since scaffolds retain only 4% of chromosomal protein they were not easily observed in intact chromosomes. Despite the controversial natures of the scaffold and the nuclear matrix these skeletal structures provide a compelling unifying model for cell cycle-dependent chromatin organization. Further experiments are required to more precisely determine how these structures may be related.

5. Matrix Protein Phosphorylation

The functions of many proteins are reversibly altered by post-translational modifications such as phosphorylation. Phosphorylation of NHP is thought to play a role in DNA replication, specific binding of proteins to DNA and transcriptional control. Phosphate levels in NHP are greatest during periods of maximal RNA synthesis (eg. Olson, 1983).

Major phosphoproteins identified in HeLa S3 nuclear matrix include lamins A, B and C and bands at 42-45k, 55k, 100k, 110k, 130k and over 200k (Henry and Hodge, 1983). The species at 42-45k are likely hnRNP core proteins. Lamin phosphorylation precedes mitosis as discussed above. Henry and Hodge (1983) detected an endogenous protein

kinase in HeLa nuclear matrix. They suggested, that generally low levels of phosphorylation of matrix polypeptides observed during mitosis may reflect a reduction in protein interactions. Song and Adolph (1983) also studied levels of NHP phosphorylation in HeLa matrices in relation to cell cycle. A major matrix phosphoprotein at 119k was found to be maximally modified during S phase. During G2 and mitosis it was over 90% dephosphorylated, with low levels of phosphate detected in this species during a subsequent G1 phase.

Major nuclear phosphoproteins in rat liver are located at 110k, 117k and 128k. Amanitin treatment caused a marked reduction in the amount of p110 in whole nuclei, with none detected in matrix or residual hnRNP fractions after 1-3h in the presence of inhibitor. This effect was reversible, with a complete recovery to control conditions observed 5h after amanitin addition (Schweiger and Kostka, 1984). The same phosphoprotein was shown to be increased 3-fold by weight after 24h conA stimulation of rat lymphocytes. In addition, labelling of newly synthesized RNA paralleled increased phosphorylation of p110 during the first 20-24h ps, implying a relationship between the two phenomena. Addition of DRB to inhibit RNA-pol 2 severely reduced the quantity and phosphorylation level of p110. Hydroxyurea inhibition of DNA replication had no observed effect on p110 (Kostka and

Schweiger, 1985). Stott and Williamson (1978b) found the major nonhistone phosphoprotein in LPS-stimulated mouse splenocytes at 115k, with a 17-fold rise in phosphorylation of pre-existing protein by 68h ps.

Clearly all of the roles played by phosphorylated NHP in the matrix have not been discovered. However there seems to be good evidence for potential control of at least RNA synthesis. The involvement of phosphoproteins in DNA replication or hormone action are less well-documented and minor matrix phosphoproteins have yet to be assigned possible roles. These species can be detected by 2-D PAGE due to an acidic shift in pI caused by phosphorylation. Phosphorylation of matrix proteins may be important in modulating protein-protein interactions and matrix "stability" during the cell cycle, as has been suggested for the lamins (Gerace and Blobel, 1981).

C. FUNCTIONS OF THE NUCLEAR MATRIX

1. Association with DNA

The nuclear matrix has been operationally defined as the residual structure remaining after extracting isolated nuclei with non-ionic detergents, exogenous or endogenous nuclease(s) and buffers of low and high ionic strength.

The quantity of DNA remaining in the matrix varies but extensive nuclease action removes 95-99% of total nuclear DNA (Berezney, 1984). It is possible to isolate residual nuclear skeletons which contain intact DNA. McCready et al (1980) treated HeLa cells grown on coverslips sequentially with buffers containing 0.5% Nonidet P-40 (NP-40) and up to 2.0M NaCl. All of the nuclear DNA was recovered and was contained in a flexible cage of RNA and protein. These "nucleoids" resemble DNA-rich nuclear matrices entrapped within a residual cytomatrix.

Previous work showed that eukaryotic DNA is arranged as supercoiled loops of average size 85kb in cultured *Drosophila* cells (Benyajati and Worcel, 1976) to 220kb in HeLa cells (Cook and Brazell, 1975). Whole-mount electron microscopy of HeLa nucleoids has shown that the associated DNA is arranged as supercoiled (ie. intact, unnicked) loops attached to the residual cage (McCready et al, 1980). Furthermore these workers used the DNA-intercalating dye ethidium bromide (EtBr) and fluorescence microscopy to

visualize the DNA "halo" which surrounded the cage. The average loop size (ie. halo diameter) was 65kbp. The halo diameter became progressively smaller as the nucleoids were digested with DNase I, confirming that the halo material was dehistonized DNA. Vogelstein and coworkers (1980) confirmed these results using 3T3 nucleoids. They observed that the halo diameter varied, depending on EtBr concentration, and concluded that the DNA loops attached to the cage were arranged as independent negative supercoils. This result was confirmed by Buongiorno-Nardelli et al (1982). Nicking of the DNA relaxed the supercoils and resulted in larger loops.

Extending these results to the nuclear matrix, Berezney and Buchholtz (1981b) prepared rat liver nuclear matrices without using nucleases. These DNA-rich nuclear matrices contained high molecular weight DNA (>500kbp), with a yield of about 65% of the total nuclear DNA. Electron microscopy of thin sections revealed a structure very similar to that of the DNA-depleted matrix, with the addition of a prominent internal DNase-sensitive meshwork. SDS-PAGE results showed virtually no difference in polypeptide content, regardless of DNA content. Extrapolating from data obtained using DNA-depleted nuclear matrices, Berezney and Buchholtz (1981a) calculated the average DNA loop size in rat liver nuclei

to be 80kbp, with approximately 125,000 loops per nucleus.

Lebkowski and Laemmli (1982a) dehistonized isolated HeLa nuclei and observed an increase in size of supercoiled loops, as expected. They showed that these loops were not only stabilized by binding of their bases to the matrix but that metalloproteins containing $\text{Cu}^{++}/\text{Ca}^{++}$ in the nuclear matrix helped to maintain loop compactness and supercoiling.

Buongiorno-Nardelli et al (1982) studied attachment of DNA supercoiled loops to interphase nuclear matrices. The mean loop size in Xenopus laevis erythrocytes and cultured cells was an identical 90kbp. They found that the fluorescent halo (ie. loop) size was directly proportional to replicon length in several animal and plant species, implying a possible connection between DNA loop binding to the nuclear matrix and DNA replication. Vogelstein et al (1982) measured the DNA loop lengths of Physarum polycephalum and human cells using the fluorescent halo technique. Replicon lengths in the same cell types were determined by fiber autoradiography. The mean loop lengths in Physarum and human cells were 36kbp and 75kbp, respectively, while replicon lengths were 37.5kbp for Physarum and 69kbp for human. Autoradiography performed after pulse-labelling of newly-replicated DNA has shown that this DNA is associated with the nuclear cage during replication. Chase periods of up to 30-60 min

result in appearance of the grain over the DNA halo, with little label remaining over the cage. Vogelstein *et al* (1980) reported that the time taken for newly replicated DNA to move through the halo (60 min) is the mean time required for replication of each replicon unit. Taken together these results imply that each supercoiled DNA loop attached to the nuclear cage or matrix consists of one replicon.

Pulse labelling of cells for one minute with [³H]-thymidine preferentially labels newly-replicated DNA. Nucleoids isolated from pulse-labelled 3T3 cells, when processed for autoradiography, have a majority of silver grains over the cage. Digestion of these preparations with DNase I or EcoRI progressively cleaves more DNA from the halo and, ultimately, the cage. As the DNA content decreases there is an enrichment for newly-replicated DNA attached to the cage (Vogelstein *et al*, 1980; 1982). As discussed above this label can be chased into the distal regions of the cage-associated loops over one hour. These data suggest that the matrix contains fixed sites for DNA replication. The replication complexes and replicating DNA are very stable, as they resist extraction by nuclease and high salt concentrations (McCready *et al*, 1980). Thus it appears that the supercoiled DNA loops may be attached to the matrix, at least partially, via DNA replication sites. Furthermore Vogelstein and coworkers (1980; 1982)

and others have developed models whereby mobile DNA loops are reeled through the static replication complexes. These models maintain the higher orders of DNA organization.

The potential involvement of the nuclear matrix in DNA replication was reported first by Berezney and Coffey (1975). During liver regeneration in rats DNA was pulse-labelled with $[^3H]$ -TdR for 1-30 min. Nuclei were isolated and fractionated by the matrix preparation procedure. DNA released in the low Mg^{++} buffer was referred to as bulk DNA; that removed by 2M NaCl was termed soluble. The remainder of the DNA was the residual matrix fraction. The bulk DNA had low specific activity for the first 10 minutes, followed by a linear increase to 30 minutes. Inversely, the matrix-associated DNA initially had a high specific activity which declined from 10-30 minutes. From these data it was concluded that DNA associated with the nuclear matrix was enriched for newly synthesized material which moved away from the DNA attachment sites with time. Expanding on these results Berezney and Buchholtz (1981b) used endogenous DNase-digested rat liver matrices and found that the bulk DNA fraction contained 75-85% of the total nuclear DNA, the soluble fraction accounted for 18-24% and the residual matrix DNA represented only 1-2% of the nuclear DNA. Using protease inhibitors in their preparations they

observed that 50% of the [3H]-TdR label was found in the matrix after a one minute pulse, while only 5% remained after a 10 minute pulse. On the other hand, the amount of [3H]-TdR in the bulk DNA fraction increased from 5% at one minute to 65% at 10 minutes. The data suggest that replication forks are attached to the matrix, with replicated DNA found progressively further from the matrix with time. These conclusions are similar to those reached with DNA-rich nuclear matrices from regenerating rat liver pulse-labelled *in vivo* with [3H]-TdR (Berezney and Buchholtz, 1981a). Short pulses (eg. 1 min) labelled small matrix-attached DNA fragments, including putative 4-5S Okazaki fragments. These short replicative intermediates were chased into larger DNA fragments (>100kbp) after a one hour pulse or chase.

Smith et al. (1984) studied the spatial distribution of DNA attachment and replication sites in rat liver nuclear matrix and pore complex-lamina. When DNA was pulse-labelled *in vivo*, before cell fractionation, 85% of the newly synthesized DNA (ie. grains) was in the interior of *in situ* nuclei or isolated nuclear matrices. Nuclei and matrices pulse-labelled during *in vitro* DNA synthesis driven with pol. α were labelled identically. Therefore localization of *in vivo* replication sites and *in vitro* synthetic sites correlate with the sites of DNA loop attachment to nuclear matrix throughout the nuclear

interior. Extraction of the matrix interior results in the removal of most *in vivo* replication and DNA loop attachment sites. Support of this work came from Van der Velden *et al.* (1984) who studied *in vitro* DNA synthesis in permeabilized cultured bovine liver cells.

Berezney's group (Basler *et al.* 1981, Berezney *et al.* 1982) hybridized residual DNA fragments (<350bp) from rat and mouse liver matrices to excess total nuclear DNA and observed no enrichment for repetitive or middle repetitive DNA sequences. They concluded that it was likely that DNA loops are not attached permanently to the matrix at the same sequence(s), implying dynamic loop attachment and matrix-bound replication complexes, rather than the opposite. These conclusions were challenged by Aelen *et al.* (1983) who used a novel approach to study the attachment of DNA replication origins and forks to the matrix during the cell cycle. Unlike regenerating rat liver the nuclei of the plasmodial slime mold Physarum show excellent synchrony during the nuclear cell cycle. Newly replicated DNA was pulse-labelled at various times during the S phase and chased to late G2. DNA labelled in mid-S phase was not retained in matrices during graded nuclease digestion of preparations in G2. However replication origins labelled at the onset of S phase were preferentially associated with matrix both during G2 as well as subsequent S phase. DNA labelled in late S phase

(ie. termination sites) was released more rapidly from matrices than bulk DNA in G2. The authors concluded that replication origins are bound to the matrix throughout the cell cycle and that the binding was likely non-random and permanent. The replitation fork likely binds to the matrix in a random transient manner. The sequence(s) of the DNA at the putative replication origins was not determined by Aelen et al (1983).

The above discussion implies an association of DNA replication complexes with the nuclear matrix. DNA polymerase alpha (pol a) is the enzyme responsible for DNA replication whereas polymerase beta (pol b) is a repair enzyme. Smith and Berezney (1980), studying normal and regenerating rat liver, observed enhanced pol a activity but control pol b activity in isolated nuclei from the latter sample. Nuclear matrix from regenerating liver contained significant amounts of pol a activity but no detectable pol b. In *vitro* DNA synthesis in normal and regenerating liver revealed minimal DNA pol b activity in isolated nuclear matrices, with a 10-fold depletion measured when compared to nuclei of cells (Smith and Berezney, 1982). The products of *in vitro* DNA pol a synthesis in matrices included large amounts of the 4-5S primary replication intermediates (Okazaki fragments). Increased pol a content in the isolated matrix and elevated *in vitro* DNA synthesis corresponded to higher pol

a levels *in vivo* during the period of induced DNA replication (12-16h post-hepatectomy). DNA pol α was enriched in the matrix 3-fold with regard to protein and 10-fold with regard to DNA content. Smith and Berezney (1982) concluded from these experiments that functional DNA replication complexes are dynamically associated with the nuclear matrix during active DNA replication. Jones and Su (1982) found a 2- to 4-fold enhancement in matrix-bound DNA pol levels (95% pol α) in SV40-infected or transformed African monkey cells, perhaps correlating with enhanced viral and/or host DNA synthesis.

Using high resolution autoradiography Smith and coworkers (1984) localized *in vivo* and *in vitro* synthesized DNA in nuclear matrices and laminae. Polymerase alpha activity was enriched within the internal matrix and depleted in the lamina fraction. The pore complex-lamina was enriched for pol b activity, with 47-52% of the total matrix activity coisolating in this nuclear subfraction. When *in vitro* DNA synthetic assays were optimized for pol α activity, 81% of grains were localized over the interiors of isolated nuclei and 72% were observed over the matrix interior. Optimizing for pol b activity resulted in the appearance of 78% of grains over the nuclear periphery and 73% of grains over the matrix periphery.

Mullenders *et al* (1982) studied DNA repair activity

after irradiation of human fibroblasts with UV. As in most earlier studies the authors detected no preferential association of DNA repair activity with the nuclear matrix. Immunofluorescent staining of chick embryo cells using an antiserum directed against DNA pol b gave weak staining over the whole *in situ* nucleus and no detectable stain in isolated matrices (Yamamoto *et al*, 1984). When anti-pol a serum was used for staining, strong nuclear staining was observed *in situ*, with several hundred homogeneously-sized granules per nucleus. The rest of the nucleus was covered by a weak uniform stain. A similar staining pattern was observed in isolated nuclear matrices. These results provide good circumstantial evidence for the existence of at least DNA pol a in the nuclease- and salt-resistant nuclear matrix.

Several groups have attempted to determine whether specific DNA sequences mediate attachment of DNA loops to the nuclear matrix. Due to the large number of loops per genome much attention has focused on repetitive and middle repetitive sequences. Bovine satellite DNA (sat-DNA) represents 15% of that genome. In matrices from cultured bovine kidney cells, with as little as 1% residual DNA, up to 60% of matrix-attached DNA is sat-DNA. This represents a 4-fold enrichment of these sequences (Matsumoto, 1981). It is not known whether replication origins are contained within this sat-DNA. Small and coworkers (1982) studied a

variety of repeated DNAs in 3 animal species with regard to attachment of DNA loops to the matrix. Mouse sat-DNA, arranged in tandem arrays at centromeres, represents 10% of the mouse genome. The residual DNA of matrices from induced Friend cells is enriched 2.6-fold for sat-DNA, indicating that 26% of matrix DNA attachment sites may be sat-DNA. If not all sat-DNA tracts are involved in DNA loop attachment this represents an underestimate. Other repeated sequences, including the mouse EcoRI repeat, are not enriched in mouse cell matrices. Alu sequences are likewise not enriched in human cell nuclear matrix. The African green monkey alpha component is a tandem repeat of nontranscribed centromeric DNA which accounts for 20% of the genome in these cells. Monkey cell matrices are depleted in this repeated sequence. The relationship between repetitive DNA sequences and nuclear matrix is clearly complex and species-dependent.

Concerned that 2M NaCl may cause sliding of DNA attachment points or precipitation of transcriptional complexes onto the matrix, Mirkovitch et al (1984) used a mild low-salt extraction to isolate nuclear "scaffolds". Drosophila histone genes are repeated tandemly (100 copies/genome), with the H1 gene separated from the H3 gene by a long AT-rich spacer. Mirkovitch et al (1984) found a highly specific DNA loop structure within this region, with one attachment site per repeat. This

attachment site occurred within the nontranscribed AT-rich spacer, which was enriched on nuclear scaffolds.

Furthermore, the region around the attachment site had a highly ordered nucleosomal arrangement, as detected by nuclease digestion. This suggests a possible NHP-mediated organization of DNA loop attachment near some repeated sequences. This hypothetical NHP may be matrix-derived.

The hsp70 genes in Drosophila code for the major heat shock protein. The authors found an attachment site in the nontranscribed spacer between the duplicated genes in control cells. Induction of heat shock (and transcription) resulted in no change in the attachment sites on the scaffold. Finer mapping of this region showed the presence of blocks of sequence homology upstream from the genes, known as X elements. Probes recognizing these AT-rich elements hybridized to at least 40 other cytogenetic loci, implying a family of attachment sites. The authors suggest these loci may be grouped into DNA loops sharing a common nuclear microenvironment. The potential importance of AT-rich sequences in attachment of DNA loops to the nuclear scaffold or matrix is presently unknown.

In an effort to detect DNA sequences possibly corresponding to replication origins, Goldberg et al (1983) synchronized 3T3 cells at the G1/S boundary. Two sequence families were found to be highly enriched in the

residual matrix DNA. The G4 repeat was 152bp long and contained three 31bp direct head-to-tail repeats. The G5 repeat was 135bp long and contained two domains of sequence homology. Domain 1 was a 63bp sequence homologous to the repetitive B1 sequence of mouse. Both G4 and G5 were similar to known eukaryotic replication origins in SV40 and papovavirus. An 11bp sequence at the end of each repeat in G4 was homologous to a large T antigen recognition sequence for a human papovavirus. Extensive potential secondary structure was detected in the G5 repeat. In addition several stretches of the 5bp viral large T antigen recognition site were present in the G5 repeat family. Goldberg et al (1983) could not determine whether these specific DNA sequences were only attached to the matrix during initiation of DNA replication (G1/S boundary). The alternative is that these potential replication origins are bound to the matrix permanently. As discussed previously, Aelen et al (1983) showed the replication origins of Physarum to be bound to the nuclear matrix throughout the nuclear cell cycle. The studies referred to above illustrate a potential problem when interpreting soluble hybridization experiments (Cot curves). Short DNA sequences which mediate DNA attachment to the matrix may not be detected. In many instances it may be necessary to use specific genetic probes in order to find enriched sequences in the

matrix (see Mirkovitch *et al.*, 1984). In addition it may be found that DNA sequences which appear to be matrix-bound in one species are not attached in the matrices of other species (eg. repetitive DNAs). Further study of eukaryotic viral replication origins (sequences and binding proteins) may be of use in searching for the corresponding structures in the host cell, specifically with regard to their attachment to the nuclear matrix.

2. Association with Transcribed Genes

In order to determine whether there is a potential role for the nuclear matrix in regulation of gene expression some workers have attempted to demonstrate an association between specific, transcriptionally-active genes and the nuclear skeleton. Often dot- or Southern-blot hybridization of residual matrix DNA with specific DNA probes is used to indicate enrichments of these sequences in matrix (vs. total nuclear) DNA. Restriction endonuclease treatments may be included in such experiments in order to more precisely determine how genes are bound to the nuclear matrix.

Early experiments by Robinson *et al.* (1982) suggested that actively transcribed genes were attached to the nuclear matrix. Using dot-blot hybridization these workers observed that residual DNA from chicken oviduct

matrix was enriched for the ovalbumin gene but not for globin gene sequences. The converse was true for chicken liver matrix. Expanding on these results Robinson and coworkers (1983) treated immature chicks with estrogen to stimulate ovalbumin gene expression and observed an enrichment of ovalbumin gene fragments in oviduct matrix, particularly the 5' and 3' nontranscribed areas of the gene. Hormone withdrawal reduced this enrichment while a second hormone treatment caused a rapid re-enrichment. Control experiments using globin gene probes (not expressed or enriched) or brain nuclear matrix (ovalbumin not enriched) showed these results were specific. Ciejek et al (1983) confirmed the above results using similar experiments with chicken oviduct matrix. Jost and Seldran (1984) used a protocol similar to that discussed above to further explore the relationship between the matrix and active genes. In laying hens vitellogenin is synthesized in the liver and the gene is enriched in liver nuclear matrices. Vitellogenin is not expressed in oviduct tissue and the gene is depleted in oviduct matrix residual DNA. Therefore it appears that hormonally-responsive genes are preferentially associated with the nuclear matrices of the target organ. In immature chicks treated with estradiol a lag phase occurs before vitellogenin synthesis. Jost and Seldran (1984) observed that the gene associates with the liver matrix before induction of mRNA synthesis is

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detected. This association is reversible. Hormone withdrawal causes a fairly rapid decrease in both transcription and gene enrichment in the liver matrix whereas secondary hormone treatment enhances vitellogenin gene enrichment and transcription. Jost and Seldran (1984) determined that the coding region of the vitellogenin gene is associated with liver matrix. In addition they found that 1.5-2kb of the 5' and 2kb of the 3' bordering regions of the gene were matrix-associated. As will be discussed later attachment of hormonally-responsive genes to the matrices of target tissues may be mediated by steroid hormone receptors.

Hentzen et al (1984) observed that after removal of 98-99% of DNA and RNA from chicken erythrocyte nuclear matrices there was significant enrichment (18- to 24-fold) of beta-globin gene sequences, even though the globin genes were not expressed. Ovalbumin gene sequences were not enriched in the erythrocyte matrix. This result may imply that potentially transcribed or developmentally regulated genes are matrix associated for long periods, regardless of their transcriptional state.

Further evidence for the attachment of active genes to the nuclear matrix has been provided using heat-shocked *Neurospora* cells. Small et al (1985) showed that the hsp70 and hsp23 genes are enriched several-fold on the matrix in control cells. Heat shock induces elevated

transcription rates of these genes (over 2-fold) without altering the levels of enrichment of these genes in the matrix. Removal of over 99% of RNA using RNase (before or after salt extraction) did not affect the results, indicating that the association of heat shock protein genes with the matrix is not (hn)RNA-mediated. These results contradict those of others, such as Kirov *et al* (1984), who contend that transcribed genes are precipitated on the matrix by salt-insoluble proteins in transcriptional complexes. The persistence of active gene associations with the matrix after mild nuclear extraction (Mirkovitch *et al*, 1984; Jackson and Cook, 1985b) weakens the argument for artifactual aggregation of DNA to the matrix.

Confirming the experiments discussed previously Small *et al* (1985) found that the Drosophila hsp70 and actin genes were preferentially attached to the matrix at several points by their 5' nontranscribed ends.

Mirkovitch *et al* (1984) also found that hsp70 genes were attached to Drosophila nuclear "scaffolds" by nontranscribed upstream sequences. These specific sequences did not change when transcription was induced during heat shock. These regions have been discussed with respect to attachment of repeated DNA sequences to the nuclear matrix.

At present it is not entirely clear how genes are

bound to the nuclear matrix during transcription. Specific sequences upstream of the genes and specific proteins (eg. steroid hormone receptors) have been implicated. Some evidence suggests that the transcriptional apparatus may bond the genes to the nuclear skeleton (Jackson and Cook, 1985b), followed by "reeling" of the DNA through the matrix sites during transcription.

3. Association with RNA/RNP

Miller *et al* (1978a) were the first to isolate a nuclear matrix fraction containing tightly-bound high molecular weight, rapidly-labelled RNA (ie. hnRNA). They prepared rat liver nuclear matrices without exposure to exogenous RNase and observed a structure quite similar to the nuclear protein matrix of Berezney and Coffey (1977), which contained almost 80% of the total hnRNA. Removal of over 95% of RNA by exogenous RNase resulted in an almost identical matrix ultrastructure. Miller *et al* (1978a) concluded that hnRNP complexes are naturally attached to the nuclear skeleton and do not represent important structural elements.

Long *et al* (1979) extracted over 95% of DNA from nuclear matrices of stimulated and unstimulated Friend erythroleukemia cells under physiological ionic

conditions. The internal matrix structure was not modified by this procedure and virtually all of the hnRNA remained undegraded and tightly attached to the matrix. Long et al (1979) concluded that nuclear RNA (snRNA, hnRNA) was attached to proteinaceous fibril cores as ribonucleoprotein (RNP) particles. Low or high ionic strength buffers did not remove this material from the residual nuclear fraction, suggesting a stable supramolecular structure. Only treatments which destroyed the matrix (eg. 8M urea, deoxycholate) could release relatively intact hnRNA. This was confirmed by van Eekelen and van Venrooij (1981) using HeLa cell nuclear matrices.

The mode of attachment of hnRNA to the matrix has been partially determined. Herman et al (1978) digested over 98% of RNA from HeLa nuclear matrices and found some unusual RNA sequences resistant to RNase digestion. Over 56% of double-stranded RNA (dsRNA) was matrix-bound, perhaps acting as hnRNP binding sites. In addition, over 50% of the nuclear poly(A) tracts of hnRNA remained firmly attached to the nuclear matrix. Subsequent incubation of nuclear matrices in 10 mM EDTA caused release of a 12S particle. Blobel (1973) observed a similar particle released after mild RNase digestion of isolated hnRNP fractions. The poly(A) was associated with a 78k polypeptide. A very basic polypeptide of molecular weight

73k is a minor component of the avian erythroblast nuclear matrix (Maundrell *et al.* 1981), suggesting that hnRNA may be bound to a nuclear skeleton via the 3' poly(A) tract and its associated polypeptide(s).

Van Eekelen and van Venrooij (1981) induced cross-linking of RNA and associated proteins by UV irradiation of intact HeLa cells or isolated HeLa nuclear matrices. In both cases they observed a tight association between hnRNA and polypeptides of 41.5 and 43kd. RNase and high-salt treatments of matrices resulted in release of polypeptides of 33-38k. Beyer *et al.* (1977) analysed hnRNP core proteins and found three groups of major polypeptides; namely classes A (32k, 34k), B (36k, 38k) and C (42k, 44k). Jacob *et al.* (1981) showed that when isolated hnRNP was extracted with 0.3M NaCl, only the class C proteins remained firmly attached. Thus it is possible that hnRNA is also tightly attached to the nuclear matrix via the class C hnRNP core proteins.

Using EM spreads of transcribing genes, Beyer and Miller (1980) concluded that nascent RNA associates with proteins, as it is transcribed, to form RNPs. Herman *et al.* (1978) concluded that nascent hnRNA attaches to HeLa nuclear matrices within 15 seconds after transcription initiation. Jackson *et al.* (1982) produced HeLa cell matrices containing unbroken, supercoiled, histone-free DNA in a flexible cage of RNA and protein. Rapidly

labelled RNA was attached to the cages, even after removal of over 95% of the DNA. Radioactive labelling of the 5' methylated caps suggested that the nascent RNA may be attached to the cages by the 5' ends of the molecules. Recent work by Jackson and Cook (1985) indicates that RNA may become attached to the nuclear matrix during transcription. HeLa cell nucleoskeletons were isolated under isotonic conditions with complete removal of DNA. High and low ionic strength buffers were avoided to reduce the possibility of artefact. Jackson and Cook found that over 80% of the total nascent RNA remained attached to the nuclear cage. RNA polymerase 2, which synthesizes hnRNA, was also attached to the HeLa nucleoskeleton. Therefore all of the major components of the transcriptional complex (ie. DNA(gene), RNA pol 2, hnRNA) are attached to the nuclear cage or skeleton. All of these components can be disrupted in hypotonic buffer (1.5M NaCl), suggesting they are parts of a single structure. Jackson and Cook (1985) propose that the nuclear skeleton is part of the active site of the transcriptional complex, spatially organizing the DNA template close to a fixed RNA pol site. As a gene passes through the RNA pol site the nascent RNA becomes attached, possibly as RNP.

The small nuclear RNAs (snRNA) are a class of uridine-rich molecules of 4-7S. They are complexed with a group of specific polypeptides to form 10S small nuclear

ribonucleoproteins (snRNPs). Some snRNA species have been strongly implicated in pre-mRNA processing (splicing) reactions (Black et al, 1985). Miller et al (1978b) isolated rat liver nuclear matrices which contained all of the major snRNA species tightly attached. In bovine lymphocyte matrix 45-80% of snRNAs remain with the matrix, after removal of 98% of DNA and 85% of the protein. These RNAs are associated with the matrix as RNP (Nakayasu et al, 1982). The snRNPs can be removed from the matrix as 10S particles by sonication (Vogelstein and Hunt, 1982) or by mild sodium deoxycholate extraction (Pogo et al, 1982), indicating that snRNPs and hnRNPs are distinct structural entities on the nuclear matrix also. Anti-Sm serum from patients with systemic lupus erythematosus (SLE) is directed against human snRNP. Immunofluorescent staining using this serum has shown the localization of snRNP within 3T3 cells, nuclei and subnuclear fractions to be the same (Vogelstein and Hunt, 1982).

In order to show that snRNA and hnRNA on the nuclear matrix interact in a sequence-specific manner, DNA-depleted HeLa nuclei were treated with long-wave UV and a psoralen compound. Hydrogen-bonded nucleic acids are cross-linked by this method. The U1 snRNA is the most abundant species paired with hnRNA (Pogo et al, 1982). This snRNA has been most strongly implicated in hnRNA splicing reactions.

SnRNA association with the nuclear matrix provides circumstantial evidence for an RNA-processing function at the nuclear skeleton. More direct evidence was provided by van Venrooij *et al* (1982) and Mariman *et al* (1982). Adenovirus type 2 (Ad2) pre-mRNA is capped, methylated, polyadenylated and spliced within the host cell nucleus, essentially like host hnRNA. Using matrix-bound polyadenylated hnRNA extracted from Ad2-infected HeLa cells hybridized to various DNA probes, it was established that virus-specific hnRNA, splicing intermediates and processed mRNAs were all attached to the nuclear matrix. The authors concluded that Ad2, and probably host, pre-mRNA was processed in association with the nuclear matrix. The various Ad2 RNAs were bound to HeLa nuclear matrix via the same polypeptides (41.5k and 43k) as host hnRNA (Mariman *et al*, 1982; van Eekelen and van Venrooij, 1981). Ad2 RNA was cross-linked to the proteins *in vivo*, before nuclear or matrix isolation, to reduce the likelihood of artefactual aggregation.

A RNase activity copurifies with DNA-free residual nuclear skeletons of rat liver and baby hamster kidney (BHK) cells (Herzberg *et al*, 1983). RNase-free DNase was used to digest DNA before salt extraction. High ionic strength solutions (2M NaCl) extracted some endogenous RNase from the residual nuclei and detergent extraction solubilized the remaining activity. The RNase activity

associated with the "matrix" may be associated with RNA processing and/or RNA binding. It is also possible that it could be involved in RNP transport via the nuclear periphery.

One potential control on gene expression lies in regulated transport of RNP from the nucleus to the cytoplasm. Since RNA is not released from isolated nuclei when the nuclear membrane is torn (Maul, 1982) or removed by detergent (Wunderlich et al, 1984) it is plausible that RNP transport involves a solid phase system as opposed to passive diffusion. This system must be potentially continuous from the nuclear interior to the peripheral pore complexes. The nuclear matrix has been proposed as the most likely structural element in this regard (Baglia and Maul, 1983; Maul, 1982; Agutter, 1984; Wunderlich et al, 1984). Such a model requires mechanisms for RNP attachment (see above), RNP release and generation of movement.

Release of RNP from the nuclear matrix might be accomplished by modification of the hnRNA-binding polypeptides, thus decreasing their affinity for RNA. Phosphorylation has been proposed as one possible mechanism (Agutter, 1984). Several nuclear proteins are phosphorylated/dephosphorylated, including those of the hnRNP. Olson (1983) reports that the class C hnRNP core proteins are the most highly phosphorylated species.

although others may also be modified. The class C polypeptides attach hnRNA to the nucleoskeleton (van Eekelen and van Venrooij, 1981). Modification of the RNA may also be involved in a release mechanism. This would require RNA excision from the matrix, followed by specific ligation.

Movement of RNPs from nucleus to cytoplasm likely occurs at the nuclear pore complexes (eg. Clawson and Smuckler, 1982; Maul, 1982; Baglia and Maul, 1983; Agutter, 1984). Here the nuclear matrix may be continuous with elements of the cytoskeleton. Fibrils traversing the pore complexes provide potential continuity between the cytoplasmic and nuclear matrices (Maul, 1982). Transport of RNP in "higher" eukaryotic systems (eg. rat liver) involves hydrolysis of ATP via nuclear membrane NTPase (Baglia and Maul, 1983; Clawson and Smuckler, 1982). Baglia and Maul (1983) found that antibodies directed against the 67k lamin B, a major component of the pore complex-lamina, inhibit both RNA translocation and NTPase activity in isolated nuclei. The lectins con A and WGA had similar effects. These authors suggest that lamin B may be a glycoprotein possessing the NTPase activity required for RNA translocation.

It is not clear what the main rate-limiting step in RNP transport is, although Wunderlich et al (1984) suggest that intranuclear matrix regulates the number of

potentially transported nuclear RNP and the number of RNP transportable at a given temperature (transport decreases as temperature is lowered). Detergent treatment of isolated Tetrahymena to remove the nuclear envelope and pore complexes had no effect on RNP transport kinetics. Nuclear RNP did not diffuse out of the stripped nuclei but remained bound to an intranuclear structure. Wunderlich et al. (1984) suggest that the nuclear matrix represents the detergent-insoluble fraction which provides temperature-dependent structural restriction to nuclear RNP transport in Tetrahymena.

4. Association with Viruses

In order to assess involvement of the nuclear matrix in virogenesis most investigators have studied DNA viruses, such as Adenovirus (Ad) and Simian Virus 40 (SV 40), where transcription of viral genes, processing of viral RNA, encapsidation and genome replication are intranuclear events.

Thin and Maizel (1977) reported that the early E3 (11k) protein of adenovirus type 2 (Ad2) was enriched about 3.5-fold in the nuclear matrix fraction. Sarnow et al. (1982) found that up to 90% of the 11k protein was resistant to 2M NaCl and nucleases in HeLa matrix. Using hamster serum against Ad-induced tumors, the 11k protein

could be immunoprecipitated from solubilized matrix polypeptides. In addition, it was observed that only one of two isoelectric variants of this protein (pI 5.4) was matrix-bound. Hodge *et al.* (1977) were able to detect three Ad-specific polypeptides: 21k (virion), 23k (pre-core) and 95k (viral 100k) in nuclear matrices prepared from infected HeLa cells using 0.5M NaCl. Presumably 2.0M NaCl may have extracted these polypeptides since others have not detected them in matrices (Sarnow *et al.*, 1982; Younghusband and Maundrell, 1982).

Deppert (1978) studied early SV 40-specific proteins in HeLa and African monkey cells infected with Ad-SV 40 hybrid virus lines and labelled with [³H]-leucine. A 28k protein was exclusively located in HeLa nuclear matrix when cells were infected with Ad2+ND1. This association was stable, as a 2h chase with unlabelled leucine indicated no change in high salt-resistant binding. In African monkey cells infected with Ad2+ND2 the 56k and 42k early SV 40-encoded polypeptides were specifically and stably matrix-bound. When HeLa cells were infected with Ad2+ND4, four species (64k, 60k, 56k, 42k) were quantitatively recovered in the matrix fraction, whereas 72k, 74k and 95k polypeptides were extracted from nuclei by 2M NaCl. Furthermore, immunofluorescent staining with antiserum against SV 40-induced tumors showed that the *in situ* nuclei and isolated nuclei and nuclear matrices of

infected cells only stained when the virus used was able to induce SV 40-specific antigens.

The large T antigen (Tag) of the papavovirus polyoma is a 94/100k protein which controls initiation of viral DNA replication and binds tightly to the origin of replication. Nuclei of 3T6 cells infected with polyoma show indirect immunofluorescence using anti-Tag serum (Buckler-White *et al.*, 1980). Isolated nuclei and nuclear matrices have similar staining patterns. The Tag bound to matrix appears kinetically different from detergent- and high salt-soluble Tag. The amount of matrix-bound Tag remains constant during the infection cycle, while soluble antigen increases in amount. The matrix-bound Tag is more phosphorylated (3.8-fold) than the soluble form. Phosphorylation is greatest when DNA synthesis is initiated, and then declines (Buckler-White *et al.*, 1980). A highly phosphorylated form of Tag also co-isolates with the nuclear matrices of SV 40-infected, or SV 40-transformed 3T3 cells (Jones and Su, 1982).

Herpes simplex virus type 1 (HSV-1) exhibits nuclear replication in BHK cells (Bibor-Hardy *et al.*, 1982). Up to 20 virus-specific polypeptides have been detected after labelling of newly synthesized proteins. Three major (155k, 87k, 81k) and 6 minor bands are especially obvious in autoradiographs. Most of these proteins are capsid-specific. Electron microscopy of matrices from

infected cells shows that capsids heavily coat the internal matrix fibrils. It is not clear whether capsid association is genuine or artifactual. Adenovirus virions do not appear to co-isolate with the matrix (Hodge et al, 1977) but mature SV 40 virions completely cover nuclear matrices of infected BSC-1 cells (Abulafia et al, 1984).

Nuclear matrices prepared from Ad5-infected HeLa cells without exogenous nucleases retain 90% of host DNA and up to 99% of viral DNA which resists extraction by nonionic detergents and 2M NaCl (Smith et al, 1985; Younghusband and Maundrell, 1982). Both of these groups report that after digestion of matrix-associated DNA with restriction endonucleases all regions of the Ad5 genome are enriched equally as digestion proceeds, suggesting nonspecific interactions with the nuclear skeleton. Younghusband and Maundrell (1982) showed by *in situ* hybridization to "cage" preparations (Pardoll et al, 1980) that Ad5 DNA binds intimately to the matrix and does not extend into the host DNA "halo". Whereas Younghusband and Maundrell (1982) found Ad5 DNA to be more nuclease-resistant than host DNA throughout infection (36h), Smith et al (1985) observed complete DNase resistance only early after infection (<4h p.i.). The latter authors also showed that newly replicating DNA is preferentially associated with the nuclear matrix after DNase digestion, suggesting that the matrix is the site of viral DNA

replication.

Buckler-White *et al* (1980) found 10% of polyoma DNA bound to 3T6 nuclear matrices. Nuclear cage preparations from SV 40-transformed 3T3 cell clones were progressively digested with DNase I by Helkin *et al* (1980); SV 40 genome sequences are enriched 5- to 7-fold when 3% of the total DNA remains with the matrix. Mouse beta-globin sequences were not enriched on the matrix, suggesting specific attachment of SV 40 genomes. The authors concluded that 1-2 copies of SV 40 genome per host genome exist near the bases of supercoiled loops attached to the matrix.

Jones and Su (1982) found that DNA pol α is enriched in nuclear matrices prepared from a variety of rodent cell lines. The polymerase activities in matrices from lytically-infected or SV 40-transformed cells are 2- to 4-fold greater than in uninfected control cell lines. In lytically-infected cells, 30-40% of polymerase activity is solubilized by sonication and co-sediments with highly phosphorylated large Tag. The polymerase activity also coprecipitates with anti-T sera. Jones and Su (1982) conclude that subnuclear complexes containing DNA pol α , Tag and likely other polypeptides are matrix-bound. If Tag binds to the origin of replication, as suggested by Jones and Su (1982), a direct and vital role for the matrix in genome replication is implied. Such an association has not been demonstrated conclusively to

date.

The majority of newly-synthesized hnRNA and pre-mRNA processing intermediates specific to SV40 are tightly bound to the nuclear matrices of infected BSC-1 (rodent) cultured cells (Ben-Ze'ev and Aloni, 1983). In addition, all, or almost all, transcriptionally active SV 40 genes are highly enriched in matrix preparations from these cells (Abulafia et al, 1984). Mariman and coworkers (1982) showed conclusively that Ad2-specific hnRNA is associated quantitatively with infected HeLa cell nuclear matrices. Pre-mRNA, processing intermediates and processed mRNAs are also bound to the nucleoskeleton. Intron sequences are not bound to the matrix and are presumably detected shortly after their excision. Ad2-specific RNA molecules can be cross-linked *in vivo* by UV irradiation to the class C hnRNP core proteins (41.5k, 43k) which also bind host cell hnRNA to the nuclear matrix. Since snRNA(P) co-isolate with the nucleoskeleton it is likely that virus RNAs are spliced while attached to the matrix.

The RNA influenza virus replicates in nuclei of susceptible cells. Nuclear matrices prepared from influenza-infected embryonic chick fibroblasts contain tightly coupled viral messenger and coding RNA strands. In spread cages over 53% of DNA is in the halo whereas greater than 85% of RNA is on the cage (Jackson et al, 1981). Thus RNA viruses appear to replicate using the

host-cell transcriptional apparatus, which is matrix-attached. The rhabdovirus Chandipura is an RNA virus which replicates in the cytoplasm. Its transcripts do not copurify with the nuclear matrix.

Taken together these data suggest that the nuclear matrix plays an active role in virogenesis of nuclear viruses. Genome replication, transcription, gene expression and encapsidation all appear to occur at sites on or very near the matrix. It would seem plausible that, as viruses and their hosts co-evolved, the most successful viral lines would use a strategy which utilized host cell synthetic pathways while inhibiting host cell use and/or regulation.

5. Association with Steroid Hormone Receptors

Steroid hormones are thought to combine with cytoplasmic receptors in responsive tissues, followed by transport of receptor-hormone complexes to the nucleus. It is believed that the complexes bind at or near responsive genes (Schimke *et al.*, 1983). In the past it was thought that hormone receptors could be extracted from isolated nuclei by 0.6M NaCl or KCl. However in many target tissues a significant amount of tissue-specific steroid hormone binding activity is resistant to solubilization by 2M salt. Barrack and Coffey (1980) showed that nuclear matrices from liver of

estrogen-treated chicks contained 60% of the total nuclear hormone binding activity. In matrix preparations from rat ventral prostate approximately 50% of androgen receptors are released by nuclease and high salt treatments (Rennie et al, 1983; Barrack and Coffey, 1980).

Steroid hormones (eg. estrogens, androgens) bind specifically to the nuclear matrix of responsive tissues; e.g., estradiol binds to matrix of rat liver and uterus, whereas dihydrotestosterone (DHT) binds only to rat ventral prostate matrices. The binding satisfies other criteria for steroid hormone binding as well. It is saturable, high affinity and heat or pronase sensitive (Barrack and Coffey, 1982).

The appearance of specific steroid hormone (receptor) binding sites in nuclei occurs in response to the appropriate hormonal stimulus. Vitellogenin is normally produced only in the livers of egg-laying hens. There are about 600 estradiol binding sites per hen liver nuclear matrix, 8-fold those in matrices from rooster livers.

Treatment of adult roosters with diethylstilbestrol induces production of vitellogenin in liver and a 12-fold increase in liver estradiol binding sites. The ventral prostate of an adult rat is responsive to androgens and DHT-specific sites are present on nuclear matrices from this tissue. Castration leads to a rapid loss of hormone responsiveness, and a rapid (reversible) loss of matrix

binding sites (Barrack and Coffey, 1982).

As discussed earlier the internal nuclear matrix of some tissues can be easily extracted, leaving only a pore complex-lamina. Treatments which cause this include endogenous protease, alkaline pH with vortexing or use of sulfhydryl blocking or reducing agents with RNase. Using the latter procedure Barrack and Coffey (1980) solubilized the internal component of matrices of rat ventral prostate tissue. This treatment resulted in extraction of up to 83% of the DHT binding sites. Similar results were obtained with hen liver estrogen binding sites. The authors conclude that there is an enrichment for steroid hormone binding sites in the internal fibrogranular nuclear matrix, although a small number of lower affinity binding sites might exist in the pore complex-lamina.

Rennie et al (1983) were the first to characterize hormone binding sites of nuclear matrix. They are able to release androgen-binding activity from nuclear matrix of rat ventral prostate by sonication or mild trypsin treatment. The released activity had a sedimentation coefficient of 3S, the same as isolated cytoplasmic androgen receptor (AR) and the binding activity was indistinguishable from previously characterized soluble AR. Rennie et al (1983) calculated that prostate nuclei contain 18,000 to 23,000 AR binding sites, 8,000 to 13,000 of which are tightly matrix-bound. Colvard and Wilson

(1984) found that isolated DHT receptors can bind *in vitro* to isolated nuclear matrices from Dunning rat prostate tumor. This binding activity could be inhibited by a proteinaceous factor which binds to active AR. The authors concluded that *in vivo* there is likely an equilibrium between active (matrix binding) and inactive (non-binding) AR, the latter favoured by coupling with the above factor. Binding of androgen would result in activation of the AR-hormone complex, followed by specific attachment to the nuclear matrix. Using a cell-free binding assay, Barrack (1983) showed that attachment of cytoplasmic ARs to prostate nuclear matrices was dependent on the presence of activated AR complexes. Barrack showed that DHT-specific, saturable, high affinity acceptors for AR are present on isolated matrix. This direct binding of ARs to matrices *in vitro* shows that the association of steroid hormone receptors with nuclear skeletons need not be due to high salt-induced precipitation.

At present the nature of the steroid hormone acceptor sites is not known. The acceptor may be a residual DNA sequence, since fragments of active genes are attached to matrices. The acceptor could also be a DNA-nonhistone protein complex. The data are consistent with either model.

As discussed previously and below, recent observations in the electron microscope suggest that the

plasma membrane, cytomatrix and nuclear matrix are interconnected. Such an arrangement, if genuine, could provide a mechanism for transport of molecules, such as hormone receptors, from the cell surface to the nucleus. This "vectorial chemomechanical coupling" (Barrack and Coffey, 1983) could allow more rapid and specific signal transduction than simple passive diffusion.

6. Nuclear Matrix-Cytomatrix Interactions

When unembedded cells are observed as whole mount preparations in scanning or transmission EM, skeletal frameworks of 3-dimensional anastomosing fiber networks in both the cytoplasm and nucleus are readily observed. The shape of the *in situ* nuclear matrix from HeLa or 3T3 cells is similar to the intact nucleus. Intranuclear 3nm fibers can be observed; these may coalesce to form fibers up to 22nm thick. The nuclear lamina is visible, as are many cytoplasmic IF which form a surrounding *in situ* network (Capco et al, 1984). When epithelial cells (Madin-Darby canine kidney (MDCK)) in culture are extracted these features are more easily seen (Fay et al, 1984). After 0.5% Triton X-100 extraction of soluble proteins, DNase and RNase digestions are performed. Elution with 0.25M ammonium sulphate leaves a nuclear matrix-intermediate filament scaffold (NM-IF) which retains the 3-D architecture of the original cells. The nuclear matrix is

continuous with IF which extend from the residual nuclear lamina. Two-dimensional PAGE of these preparations suggests the presence of vimentin and actin in addition to matrix polypeptides. Blotting analysis (1-D) confirms the presence of abundant vimentin and cytokeratins in preparations of NM-IF from MCDK cells.

These results with extracted cells show the intimate association between the nuclear matrix and cytoskeletal elements. This association has also been demonstrated with isolated nuclei and matrices. When Staufenbiel and Deppert (1982) isolated nuclei from HeLa cells they found that IF proteins were coisolated. Immunofluorescent staining of vimentin and cytokeratins showed that IF networks were attached to single nuclei, often collapsed on the nuclear surface. Several laboratories have found an intimate association between the nuclear membrane and IF (eg. Woodcock, 1980). Actin is a major component of isolated bovine lymphocyte nuclear matrix. Two-

dimensional PAGE has shown the presence of three isoforms of actin in these structures. The authenticity of actin was confirmed by its amino acid composition and trypsin insensitivity (Nakayasu and Ueda, 1983).

These experiments raise two important considerations regarding study of the isolated nuclear matrix. First, the extent of cytoplasmic contamination of nuclear (matrix) preparations becomes visible most easily by 2-D

PAGE analysis of proteins. Because cytoplasmic filaments often collapse onto the nuclear surface during isolation, in solution the residual nuclear envelope may be an aggregate of the lamina, IF and actin. Thus contamination of this type may not be readily visible even in the EM. Second, nuclear and cytoplasmic skeletons may interact functionally *in vivo*, representing two highly specialized but interconnecting networks. The nuclear matrix differs from the cytomatrix in numerous ways. The former has a different structural organization, morphology and polypeptide composition, denser anastomosing network and greater stability in high ionic strength solutions than microtubules, microtrabeculae or intact microfilaments in the cytomatrix. However, the IF is a major cytoskeletal system in the cell which is high salt resistant. In addition, extensive sequence homologies exist between nuclear lamins and IF proteins (McKeon *et al*, 1986). Functional and structural relationships between the nuclear matrix and cytoskeleton are not well characterized. As discussed above, RNP transport, virogenesis and steroid hormone receptor function (among others) may be phenomena which require direct connection between these skeletal components of eukaryotic cells.

CHAPTER ONE

ULTRASTRUCTURE AND POLYPEPTIDE COMPOSITION OF
LYMPHOCYTE NUCLEAR MATRIX

INTRODUCTION

The interphase nucleus is a complex structure which displays a wide variety of morphologies in different cell types or in response to metabolic activation. Differences occur in nuclear volume and shape, as well as amount and distribution of condensed chromatin and interchromatinic material. Nuclei in mature and undifferentiated avian erythrocytes represent two extreme versions of eukaryotic nuclei (Lafond et al, 1983; Dardick and Setterfield, 1976). Lymphocytes are another example of cells with diverse nuclear structures. In response to mitogenic stimulation by antigens or lectins (eg. con A), lymphocyte nuclei are extensively remodelled; this involves both condensed chromatin and interchromatinic material (Setterfield et al, 1983a; 1985). Hence they provide a suitable system for studying factors which control interphase nuclear organization.

There have been proposals recently of large-scale organization of condensed chromatin distributions and nuclear structure (eg. Murray and Davies, 1979; Agard and Sedat, 1983). These models suggest that intrachromatin interactions maintain distinct nuclear morphologies. Alternatively it has been proposed that nonhistone proteins play essential roles in ordering nuclei. A skeletal nuclear framework, the nuclear matrix, is

composed of NHP and has been implicated in structural and metabolic control in interphase nuclei (see Berezney, 1984 for review).

An early objective of this study was to observe nuclear morphological changes in con A-stimulated lymphocytes by EM and to determine whether the structural changes which occur in nuclei *in situ* are maintained in isolated matrices (Setterfield et al, 1985; 1983a). Due to the large number of functions ascribed to the matrix, nuclear fractions were observed by EM during nuclear matrix isolation to identify the effects on structure of each extraction step and to determine whether nonchromatin components were randomized during NM preparation.

Nonhistone protein components of lymphocyte nuclear matrices were resolved by 2-D electrophoresis, using both equilibrium and nonequilibrium gel systems. Differences in NHP content between unstimulated and stimulated nuclear matrices were determined as cells progressed from G0 through G1 and S phases of the cell cycle, in an attempt to correlate compositional changes in nuclear matrix with metabolic activation and/or structural elaboration.

MATERIALS AND METHODS

A. Isolation and Culture of Lymphocytes

Retropharyngeal lymph nodes were excised from freshly killed cows at a local meat packing plant and placed on ice immediately until used. Fat and muscle surrounding the node were removed prior to surface sterilization in 70% ethanol (15-30 sec). Sterilized nodes were pinned to a support with the concave side facing down. An incision was made down the length of the node and tissue inside was removed using a scalpel. This tissue was placed on a fine wire screen in a 60mm petri dish containing 4-5 ml RPMI 1640 medium. After mincing the tissue with a scalpel a sterile rubber stopper was used to crush material on the screen, releasing lymphocytes. The stopper and screen were rinsed with medium and the cells were gently suspended using a pipette. Large tissue fragments and connective tissue remained on the screen.

The suspended cells were transferred to a sterile 50 ml conical centrifuge tube and centrifuged at 600 xg for 10 min. The supernatant and floating insoluble fatty material were discarded and the pellet was resuspended in 20 ml of medium with antibiotics (100 U/ml penicillin, 100 ug/ml streptomycin). The cells were centrifuged as above and the washing procedure was repeated at least twice more.

Cell number was determined using a hemocytometer; routinely 2-4 billion cells were isolated from each node. Cell viability was determined by trypan-blue exclusion. A small volume of cells in complete medium (RPMI 1640 supplemented with 10% foetal bovine serum and antibiotics) was added to 9 volumes of 0.05% trypan blue (w/v) in 0.15M NaCl. Routinely viability values greater than 95% were obtained after isolation from the nodes. Lymphocytes were then suspended in complete medium at a concentration of 5 million/ml and cultured in rectangular plastic culture flasks at 37°C. After 24h they were supplemented with complete medium (10% of original volume). Lymphocytes were mitogenically stimulated with concanavalin A (conA) at a concentration of 5 µg/ml.

B. Isolation of Nuclei and Nuclear Matrices

Lymphocytes in medium were harvested by centrifuging at 600 xg for 5-10 min. The cells were resuspended in STM buffer (0.25M RNase-free sucrose (Bio Rad), 10mM Tris-HCl, pH 7.4, 25mM KCl, 2mM MgCl₂) and centrifuged for 5 min at 600 xg. All subsequent solutions contained 1mM phenylmethylsulfonylfluoride (PMSF; Sigma), 1µg/ml soybean trypsin inhibitor (STI; Sigma) and 0.5mM sodium tetrathionate (NaTT; Fluka) to inhibit endogenous proteases.

The washed cells were resuspended in STM to a

concentration of no more than 10^8 /ml. Triton X-100 (TX-100) was added to a final concentration of 0.5% (v/v) and the cells were broken using 8-12 strokes of a tight-fitting Dounce homogenizer. The homogenate was diluted with one volume of STM without TX-100 and the nuclei were gently pelleted at 200 xg for 5 min. The pelleted nuclei were resuspended in STM and centrifuged again. Washed nuclei were examined by phase contrast microscopy to determine the extent of cytoplasmic contamination. If necessary the homogenization and wash steps were repeated to remove visible cytoplasmic 'tags'.

All steps in the isolation of nuclear matrices were done at 4°C , unless otherwise noted. Centrifugation steps were 200-400 xg for 5 min. Washed nuclei were resuspended in STM buffer (10^8 /ml) containing 10 ug/ml DNase 1 (Worthington). The nuclei were incubated for 10 min at room temperature after which they were pelleted, resuspended in low Mg buffer (LMB; 10mM Tris-HCl, pH 7.4, 0.2mM MgCl_2) and incubated for 20 min on ice. The salt concentration was gradually increased to 1M by dropwise addition of 1 volume of 2M high salt buffer (2M HSB; 2.0M NaCl in LMB). After 20 min the salt concentration was increased to 2M by dropwise addition of 1 volume of 4M high salt buffer (4M HSB; 4.0M NaCl in LMB). After 20 min the salt-treated nuclei were pelleted. Pelleted nuclei were resuspended in LMB containing 50ug/ml DNase 1. After

30 min at room temperature the residual nuclear matrices were centrifuged and the pellet was washed with LMB.

C. Electron Microscopy

Samples of cells, nuclei or ~~extra~~cted nuclei were fixed for 1-2h on ice in a solution containing 50mM sodium cacodylate, pH 7.2, 2% glutaraldehyde, 15 mM NaCl and 5mM MgCl₂. After a wash in 50mM sodium cacodylate, pH 7.2, samples were post-fixed for 1 hour in 1% osmium tetroxide in the same buffer. The samples were washed and dehydrated through a graded alcohol series before embedding in Spurr's resin (Polysciences). Silver-grey to silver sections, cut with a diamond knife, were mounted on formvar-coated copper grids. The sections were stained for 2-3 hours in 2% uranyl acetate in 50% ethanol, followed by 15 min in aqueous lead citrate. A Siemens 101 electron microscope (80kV) was used for observation and photomicrography.

D. Electrophoresis

Pelleted samples for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) were suspended in distilled water, frozen at -80 C and lyophilized in a Virtis Uni-Trap freeze dryer. Soluble or salt-extracted protein samples in aqueous solution were precipitated by 3-4 volumes of 95% ethanol (overnight at

-20°C), pelleted at 1500 xg for 10 min, suspended in distilled water and processed as above.

For one-dimensional electrophoresis, lyophilized proteins (in 15 ml plastic conical tubes) were solubilized in 50-100 μ l of sample buffer (20mM Tris-HCl, pH 6.8, 10% glycerol (w/v), 2.3% SDS (w/v), 5% fresh mercaptoethanol) by sonication for 5-10 min in a Branson sonicator bath at room temperature and boiling for 1 min in a water bath before loading onto the gel. Aliquots containing 20-40 μ g protein were loaded into 4mm-wide sample wells cast into the 5% stacking gel (made up in 125mM Tris-HCl, pH 6.8, 0.1% (w/v) SDS, 2mM EDTA). The 90 x 150 x 0.75mm slab separating gel was 10% acrylamide (made up in 375mM Tris-HCl, pH 8.8, 0.1% SDS, 2mM EDTA). Catalysts in the stacking and separating gels were ammonium persulfate (0.05% and 0.1%, respectively) and TEMED (diluted 1:100; Bio-Rad). Tube gels had identical acrylamide, buffer and catalyst concentrations. Samples were electrophoresed at 60V through the stacking gel and 120-180V through the separating gel. Bromophenol blue (0.02%) was added to the samples before loading to indicate the position of the electrophoresis front.

Gels were stained overnight in 0.05% Coomassie brilliant blue R in 10% acetic acid (v/v) and 25% methanol (v/v). Destaining consisted of a distilled water rinse, followed by several changes of 10% acetic acid in 25%

methanol over several hours. Gels were stored in 10% acetic acid.

Two-dimensional electrophoresis was performed according to the procedure described by Peters and Comings (1980). Lyophilized samples were solubilized by sonication in 2% SDS (w/v), 5mM EDTA and 5% fresh 2-mercaptoethanol in distilled water. Pure Nonidet P-40 (NP-40) was added to a final concentration of 10% (v/v). After 5-10 min of further sonication and boiling 1 min to dissolve the sample completely, urea (80 mg/ml) and ampholytes (2% v/v; Bio-Rad) were added.

For 2-D equilibrium electrophoresis samples were isofocussed to equilibrium in 3.2mm x 19cm gels (see Appendix 1). Four isofocussing gels were overlaid on the second dimension gel and electrophoresed simultaneously on the 20 x 76 x 0.15cm slab gel containing 0.1% (w/v) SDS for 20-24 hours at 60V (see Appendix 2). One isofocussing gel containing pure protein standards was routinely included in each run (see below).

For 2-D nonequilibrium electrophoresis samples were first separated in 3.2mm x 19cm gels containing different ampholyte mixtures and under different running conditions (summarized in Appendix 1). Electrophoresis in the second dimension was the same as for equilibrium gels.

After electrophoresis the slab gel was cut into four 20 x 19cm gels, stained and destained as for 1-D gels.

For increased staining sensitivity gels were completely destained and restained with silver, using a procedure modified from Sammons et al (1981) and described in Appendix 3.

Coomassie blue was used to stain the most abundant polypeptides in gels. Less abundant proteins were detected after subsequent destaining and silver staining of the same gels. The silver stain detected proteins at least one order of magnitude less concentrated.

As 2-D PAGE gels can be very complex a scheme devised by Peters and Comings (1980) to describe the gels was used. This involved use of a set of standard proteins with known MW and pI (Appendix 4) which was separated on one of four slab gels used in each experiment. Use of these standards made it possible to create a grid scheme which allowed simple classification of all spots. For example, a protein with MW 61.5k and pI 6.5 would lie in the quadrant bounded by actin (A; pI 6.1) and bovine serum albumin (B; pI 6.7). Hence it would be designated 61.5 B-A. A protein of MW 48k and pI 7.3 would be denoted as 48C as it would lie near carbonic anhydrase (C; pI 7.4).

To overcome difficulties in detecting protein spots in photographs of the 2-D gels, overlay drawings were made of each photograph. In general the most abundant proteins were easily resolved in photographs of acceptable contrast. Weaker spots observed in gels or negatives were

often not seen in these photographs. Using a range of photographic exposures weak polypeptide spots were usually depicted as smaller spots in overlay drawings.

RESULTS

A. MORPHOLOGY OF CELLS AND NUCLEAR FRACTIONS

1. Concanavalin A Stimulation of Bovine Lymphocytes

Observing light microscope sections of lymphocytes it is possible to determine the nuclear morphotype frequency (Table 1). Unstimulated samples of bovine lymphocytes consisted of 98% type 1 (unstimulated) and 2% type 2 (partially stimulated) cells. After 36h cultures containing 5ug/ml conA had 12% type 1 cells, 40% type 2 and 48% type 3 cells, the latter representing the lymphoblast population. At 48h these proportions were nearly the same, with slightly fewer type 1 cells (likely due to cell death). The response of bovine lymphocytes to conA is similar to that of human or mouse cells (eg. Setterfield *et al*, 1983a) except that there are fewer type 1 cells after 24-36h of culture.

Lymphocytes isolated from freshly excised bovine retropharyngeal lymph nodes had the typical morphology observed in other species (fig. 1a). Based on terminology used previously (Setterfield *et al*, 1983), these small

quiescent lymphocytes are referred to as type 1. The cells were small (5-6 μ m diameter) and had simple cytoplasm; in general very few organelles were observed in EM sections. The cell center, often located near a nuclear cleft, contained a centriole pair with a few short radiating microtubules. Rarely a mitochondrion was also be observed in this area. Endoplasmic reticulum was virtually absent from these cells. The nuclei of these lymphocytes had a typical appearance as well (fig. 1a). Very few nuclear pores were observed, and these were situated between large masses of peripheral heterochromatin. There was no obvious nuclear lamina situated beneath the double nuclear membrane. Instead the chromatin next to the inner nuclear membrane appeared to be highly oriented, with the nucleosomes apparently arranged parallel to the membrane in a zone almost 60nm thick. The peripheral heterochromatin was tightly compacted and in very large clumps while in the nuclear interior there were additional smaller heterochromatin clumps. In addition, in the interchromatinic area there was an amorphous, primarily granular, material present. At higher magnification (fig. 1b) interchromatinic fibrils were observed in two size classes; namely, 2-3nm diameter and 5-6nm diameter. In general the interchromatinic space was not well-differentiated ultrastructurally in type 1 cells.

Addition of concanavalin A to cultures of bovine lymphocytes resulted in a number of morphological changes similar to those described previously for mouse and human cells (Setterfield et al, 1983). Within the first 12-13 hours the cells became noticeably larger (diameter 6-7 μ m; fig. 1c) and an increased number of cytoplasmic organelles, primarily mitochondria, appeared near the cell center. This class of lymphocytes is termed "type 2". The nuclei in these cells were substantially different from those in type 1 cells. Type 2 nuclei were larger in diameter, often had a smoother outline, the double membranes had a sharper appearance and there were more numerous pores, again situated between peripheral heterochromatin clumps. Between the inner nuclear membrane and the peripheral heterochromatin a presumed nuclear lamina was observed (fig. 1c). This was a dense, amorphous, heavily stained structure, approximately 30nm thick, underlying the entire nuclear membrane except where pores occurred. The heterochromatin in type 2 nuclei was also different from that in unstimulated cells. In general, the chromatin fibrils appeared less compact and there were more numerous discrete internal clumps.

The interchromatin space in type 2 nuclei had a more open appearance than in type 1 (fig. 1d) and numerous fibrils (5-7nm diameter) and granules (12-25nm diameter) were obvious. In addition a lightly-stained wispy

material was occasionally observed in this region, interspersed between the fibrogranular material. Sections of type 2 nuclei often contained small nucleoli with visible granular components, indicating synthesis and processing of rRNA. Nuclear bodies (NB) were prevalent in type 2 nuclei. These were as large as 2 μ m in diameter and consisted of dense fibrils (2-6nm diameter). In some cases 30-50nm granules were observed near the cores of NB. In several instances fibrils radiated from the NB making apparent connections with other interchromatin structures (fig. 2). Bovine lymphocyte NB seem to have a consistent morphology, different in detail from such structures in other species (Chaly et al, 1983).

By 24-30h ps, as bovine lymphocytes prepared for DNA replication, the cells and nuclei enlarged several-fold and underwent more structural changes, to reach the type 3, or lymphoblast stage (fig. 1e). There was a great elaboration of cytoskeletal elements, including microtubules radiating from the cell center and numerous fibrils (5-10nm diameter) near the plasma membrane and surrounding the nucleus.

The nuclear membranes of type 3 cells were again quite distinct, with more numerous pores and a more strongly stained lamina. The arrangement of heterochromatin in larger nuclei changed considerably with minimal amounts near the membrane and many small internal

clumps. This gave the nucleus a more open or empty appearance. However, situated between the heterochromatin masses was an extensive network of nonchromatin nuclear structures, including numerous 6nm fibrils interspersed between 12-25nm diameter granules. As well there were large amounts of the lightly-staining amorphous material seen in type 2 nuclei. NB similar in morphology to those of type 2 nuclei were another interchromatin component of type 3 nuclei (fig. 2). The nucleoli of type 3 cells were extensive and contained well-differentiated areas of fibrillar and granular components, in addition to numerous lacunae.

2. Isolation of Nuclear Matrix

Nuclei were isolated in a neutral pH, isotonic buffer to reduce structural changes due to charge or ionic effects. Optimal recovery of clean nuclei with relatively intact structure was obtained using a nonionic detergent (0.25 to 0.5% (v/v) Triton X-100) and a Dounce homogenizer as found by Hall (1982). Use of alternate detergents, higher or lower concentrations of Triton or more rigorous homogenization resulted in reduced nuclear "purity" and/or integrity. These isolated nuclei, the starting point for nuclear matrix isolation, were termed Triton nuclei.

TABLE 1 - Frequency of Nuclear Morphotypes After
ConA Addition

Hours p.s	Nuclear Morphotype (%)		
	1	2	3
0	98	2	0
12	79	21	0
18	44	40	16
24	22	42	36
36	12	40	48

Morphotypes were determined by observation of toluidine blue-stained thin sections of pelleted cells in the light microscope. 200 cells were counted at each time p.s.

Figure 1a-f. Electron micrographs of bovine lymph node lymphocytes from a population 48h ps with con A.

Figure 1a. An unstimulated (type 1) bovine lymphocyte. Note large clumps of condensed chromatin (C) and minimal interchromatinic material (i). Bar = 1.0um. Figure 1b. High magnification of nucleus in Fig. 1a. The electron dense interchromatinic material is primarily granular. Bar = 0.25um.

Figure 1c. A partially stimulated (type 2) bovine lymphocyte. Nuclear volume is increased and there is more IM (i). Note bundle of cytoplasmic filaments near nucleus (arrow). Nu indicates nucleolus. Bar = 1.0um. Figure 1d. High magnification of nucleus in Fig. 1c. Note granular appearance of interchromatin space and few fibrils (f). Bar = 0.25um.

Figure 1e. A large, fully stimulated (type 3) lymphoblast. Note increased volume of cytoplasm and enlarged nucleus with many smaller condensed chromatin clumps (C) and electron-transparent IM. Ribosomes are found in polysomes (arrowheads). Bar = 1.0um. Figure 1f. High magnification micrograph of type 3 nucleus in Fig. 1e. Note abundant IM fibrils (arrows). Bar = 1.0um.

Figures 2a-d. Electron micrographs of nuclear bodies observed in type 3 bovine lymphocyte nuclei. C - condensed chromatin; Nu - nucleolus; i - interchromatinic material.

Figure 2a. Nuclear body consisting of a core containing large granules (arrow), fine fibrillar corona (F) and electron-transparent halo (arrowhead). Note apparent connections to fibrogranular IM. Bar = 0.5 μ m.

Figure 2b. A fibrillar nuclear body lacking a granular core. Note proximity of condensed chromatin clumps and nucleolus. Bar = 0.5 μ m.

Figure 2c. High magnification of a NB with a granular core. Very fine fibrils traverse clear halo and connect to IM. "Granules" (arrows) appear to be tightly packed fibrillar structures. Bar = 0.25 μ m.

Figure 2d. High magnification view of a fibrillar NB. Note connections to IM and chromatin clumps. Bar = 0.25 μ m.

In order to assess the success of nuclear isolation techniques fractions were embedded, sectioned and examined by electron microscope. The Triton method of nuclear isolation used gave the best compromise between retention of nuclear structure and lack of cytoplasmic contamination. In general very few cytoplasmic "tags" or unbroken cells were observed (fig. 3). Unlike mouse or human lymphocyte nuclei, bovine nuclei sometimes had cytoplasmic filaments attached or nearby (fig. 3). In some cases these 5-6nm filaments trapped cytoplasmic materials, such as ribosomes.

Triton nuclei were found to have retained their general size, shape and internal structure with little or no gross rearrangement. For example clefting of nuclei was preserved in the absence of cytoplasmic components. Only morphotype 3 nuclei were found in nuclear preparations from unstimulated cells while types 1, 2 and 3 were present in isolates from stimulated cells. Some structural alterations were, however, visible after isolation. In many Triton nuclei it appeared that the outer nuclear membrane, at least, was removed by the detergent. The presumed nuclear lamina was not as distinct in any morphotype as observed *in situ*. Both the peripheral and internal heterochromatin clumps seemed to be more compact than observed *in situ*. The clumps of heterochromatin appeared somewhat larger in isolated

nuclei although the relative differences in clump sizes in types 1, 2 and 3 nuclei were maintained (refer to figs. 3, 4).

The interchromatin space of Triton nuclei from type 1 cells consisted primarily of granules, with very few fibrils (fig. 3b). This is consistent with the more granular nature of this area *in situ* (fig. 1b). Triton nuclei from types 2 and 3 cells contained relatively greater amounts of fibrillar material, as expected (figs. 3, 4). However, in general the interchromatinic spaces of Triton nuclei appeared to be less electron-dense than in nuclei of intact cells; the fibrogranular material was not present in the same quantities seen in intact cells. It is not known if some interchromatinic material was lost during nuclear isolation; other possible reasons for the reduced electron density will be discussed later. Nuclear bodies and nucleoli remained intact in Triton nuclei with virtually identical morphologies to structures *in situ*, although NB were smaller.

In order to effectively extract chromatin from Triton nuclei it was necessary to predigest with a low concentration of DNase 1. Failure to do this led to irreversible aggregation of nuclei in subsequent steps. The DNase treatment itself had only minor structural effect at the microscopic level (fig. 5).

Figure 3a-d. Electron micrographs of Triton nuclei from bovine lymphocytes.

Figure 3a. Isolated Triton nucleus from a type 1 cell. Nuclear cleft (arrow) is preserved. Most heterochromatin (C) is located in clumps at the nuclear periphery. Bar = 0.5 μ m.

Figure 3b. High magnification micrograph of the Triton nucleus in Fig. 3a. IM is less electron-dense than in situ and is primarily granular. Bar = 0.5 μ m.

Figure 3c. Isolated Triton nucleus from a type 2 lymphocyte. Portions of outer nuclear membrane have been preserved (arrowheads). Heterochromatin texture and arrangement resembles that in situ. Bar = 0.5 μ m.

Figure 3d. An enlarged area of the nucleus in Fig. 3c. IM consists of dense 12-25nm granules and 3-6nm fibrils. Note large nucleolus (Nu) with fibrillar (f) and granular (g) zones. C - condensed chromatin. Bar = 0.5 μ m.

Figures 4a-d. Electron micrographs of Triton nuclei from bovine lymphocytes.

Figure 4a. Electron micrograph of a type 2 Triton nucleus. Bar = 1.0um.

Figure 4b. High magnification micrograph of a portion of the nucleus in Fig. 4a. A fibrillar NB is connected to the fibrogranular IM. Note condensed texture of heterochromatin, as compared to Fig. 1d. Bar = 0.5um.

Figure 4c. Electron micrograph of a large type 3 Triton nucleus, with elaborate nucleolus (Nu) and minimal peripheral heterochromatin. Large numbers of 5-10nm filaments lie between adjacent nuclei (arrows). Bar = 1.0um.

Figure 4d. Enlarged portion of Fig. 4c. Extranuclear filaments are clearly resolved. Nucleolus contains well-defined fibrillar and granular zones. NB with granular core makes connections to fibrogranular IM. Bar = 0.5um.

Figure 5. Electron micrographs of DNase 1-treated nuclei from bovine lymphocytes. Heterochromatin is highly condensed and less granular than in previous structures. Amounts of IM appear reduced.

Figure 5a. Type 1 DNase nucleus. Bar = 0.5 μ m.

Figure 5b. High magnification micrograph of nucleus in Fig. 5a. Note more fibrillar nature of IM and proximity of extranuclear filaments. Bar = 0.5 μ m.

Figure 5c. A type 2 DNase nucleus. The nucleolus (Nu) is surrounded by condensed chromatin. Many 5-10nm extranuclear filaments are present. Bar = 0.5 μ m.

Figure 5d. Magnified view of nucleus in Fig. 5c, showing interchromatinic fibrils (if) and granules (ig) and attachments to a NB (arrows). Bar = 0.5 μ m.

Figure 5e. A type 3 DNase nucleus. Extranuclear filaments may be associated with a nuclear pore (arrowhead). Some ribosomes remain associated with cytoplasmic filaments (arrow). Bar = 0.5 μ m.

Figure 5f. High magnification micrograph of the DNase nucleus in Fig. 5e. Note extraction of nuclear membrane(s) and projection of nuclear pores from the nuclear surface (arrows). Bar = 0.5 μ m.

As in undigested nuclei the condensed chromatin was highly compacted, although it was more uniform and less granular in DNase nuclei. The nucleolus changed little in structure except that lacunae appeared empty. Nuclear bodies remained unchanged in size and morphology after DNase treatment. The interchromatin areas were also unchanged, containing the fibrogranular material and lightly staining amorphous material observed in *in situ* and isolated nuclei. Some DNase 1-treated nuclei remained surrounded by 6-10nm cytoplasmic filaments which were also observed around Triton nuclei (fig. 5e).

A buffer containing 0.2mM MgCl₂ (LMB) was used to extract DNA, histones and some NHP from the DNase-digested nuclei. The residual nuclei (LMB nuclei) after this treatment had a strikingly different morphology from Triton nuclei. The nuclear periphery had a granular appearance (figs. 6,7,11), residual pores were difficult to resolve and there was no discernible (hetero)chromatin pattern. Large electron-transparent spaces beside residual nucleoli and/or residual nuclear membranes may have represented regions previously containing condensed chromatin. These were most obvious in LMB nuclei from type 1 cells. Cross sections of some +con A LMB nuclei appeared to be virtually full of fibrogranular material, with no large open intranuclear spaces seen. The "interchromatin material" present in these spheres seemed

to have a higher concentration of 4-5nm fibrils and lightly-stained amorphous material than seen in Triton nuclei. Granules in this fraction were 20-40nm in diameter. Residual NB and nucleoli appeared to be relatively intact in LMB nuclei. Several possible connections between NBs and the intranuclear "matrix" were clearly visible (figs. 6,7).

The extranuclear filaments previously described were resistant to extraction by low ionic strength buffer and in some cases large amounts of filamentous material were observed between residual nuclei (figs. 6,7).

Treatment of LMB nuclei with increasing concentrations of NaCl resulted in further modifications and/or extraction of material. As the salt concentration was gradually increased to M NaCl the residual nuclei (M HSB nuclei) seemed to become less electron dense (or stained less), resulting in slightly less clarity of structural detail. The diameters of the M HSB nuclei decreased about 30% from Triton nuclear values (Table 2).

The nuclear periphery of M HSB nuclei appeared slightly sharper and less granular than in LMB nuclei. The amorphous residual lamina was 10-25nm thick and resembled the in situ structure (figs. 8,9,11). In addition some nuclear pores were visible, particularly in tangential or glancing sections.

TABLE 2 - Diameters of Nuclei and NB from Bovine
Lymphocytes

Sample	Nuclear Diameter (μm)	NB Diameter (μm)
In situ	5.8 ± 0.9	1.1 ± 0.2
Triton	$5.3 \pm 0.7^*$	0.7 ± 0.1
DNase 1	4.4 ± 0.7	0.7 ± 0.1
LMB	4.3 ± 0.3	0.6 ± 0.1
M HSB	2.9 ± 0.4	0.5 ± 0.1
2M HSB	2.9 ± 0.5	0.5 ± 0.1
Matrix	2.5 ± 0.4	0.5 ± 0.1

Diameters were determined from 10-30 electron micrographs of types 2 and 3 cells, nuclei or extracted nuclear fractions. Values given $\pm$ std. deviation.

Figure 6. Low Mg^{++} buffer (LMB)-extracted nuclei from bovine lymphocytes. Note absence of heterochromatin, granular appearance of nuclear periphery and lack of obvious nuclear pores.

Figure 6a. Overview of LMB nucleus preparation, showing relatively homogeneous appearance of structures in con A-stimulated sample. Large empty spaces occur near residual nucleoli (RNu) and residual nuclear envelope (RNE). Many extranuclear filaments lie between LMB nuclei (arrows). Bar = 1.0 μ m.

Figure 6b. A type 1 or type 2 LMB nucleus. Note large empty spaces near the residual nuclear envelope. A small nuclear cleft is present (arrow). Bar = 1.00 μ m. (see also fig. 11).

Figure 6c. LMB nucleus from a stimulated lymphocyte. Empty spaces are relatively small and widespread. Small fibrillar NB (arrowheads) are attached to abundant fibrogranular material. A residual nucleolus contains well-defined fibrillar and granular areas. Bar = 1.0 μ m.

Figure 7. High magnification electron micrographs of LMB nuclei. Extensive fibrogranular networks contain amorphous lightly stained material (arrows). The 20-40nm granules appear to be continuous. Fine fibrils from residual NB traverse clear halo and connect to IM (arrowheads). Bar = 0.25um.

Figure 7a. Note homogeneous granular appearance of residual nuclear envelope (RNE) which lacks identifiable pores. Bar = 0.25um

Figure 7b. Large empty space exists near residual nuclear envelope (RNE). Patches of apparently aggregated material exist in some LMB nuclei (arrows). Bar = 0.25um.

Figure 8. Electron micrographs of M HSB nuclei.

Figure 8a. Overview of M HSB-extracted nuclei, showing compact structure and presence of extensive networks of extranuclear filaments between residual nuclei. Bar = 1.0 μ m.

Figure 8b. High magnification electron micrograph of the central M HSB nucleus in Fig. 8a. The dense intranuclear network contains numerous 10-20nm granules and 4-6nm fibrils. The filamentous residual NB (RNB) appears highly condensed. Bar = 0.25 μ m.

Figure 9. Electron micrographs of M₂ HSB-extracted nuclei. The residual nuclear envelope is sharper than in LMB nuclei and the IM is less granular than Triton or LMB nuclei.

Figure 9a. Large empty spaces occur near the residual nuclear envelope (RNE) and residual nucleolus (RNU; arrows). Clumps of interchromatin granules are visible in M HSB nuclei (arrowheads). The elaborate intranuclear reticulum extends throughout HSB nucleus, connecting RNE, RNU and RNB. Bar = 1.0um.

Figure 9b. High magnification micrograph of the HSB nucleus in Fig. 9a, showing empty spaces, sharper RNE and numerous intranuclear fibrils (arrow). Filaments connect a slightly condensed residual NB (RNB) to the IM. Bar = 0.25um.

Figure 9c. A M HSB nucleus with many smaller empty spaces, condensed RNB and RNU. Many 5-10nm filaments lie outside the extracted nucleus. Bar = 1.0um.

Figure 9d. High magnification micrograph of the extracted nucleus in Fig. 9c. The intranuclear network contains large amounts of fine fibrillar material (arrows). Extranuclear filaments are clearly resolved (arrowheads). Bar = 0.25um.

In some M HSB nuclei (particularly type 1) open areas of various sizes remained, perhaps where condensed chromatin had been. In others no visible open areas were observed in sections. The internal material of M HSB nuclei contained many more 2-4nm fibrils and amorphous light staining components than observed in Triton or LHB nuclei. The 10-20nm interchromatin granules were not as sharp in outline as in previous structures. Nuclear bodies present in M HSB nuclei retained their overall morphology despite their smaller sizes. Nucleoli retained their overall shape but appeared to have a more granular appearance than in earlier fractions. Nucleolar lacunae were empty and seemed to be relatively large.

Many 4-6nm extranuclear filaments resisted M NaCl extraction and were still associated with the M HSB nuclei.

Increasing the concentration of NaCl to 2M had relatively little effect on morphology of the salt-resistant nuclear fraction (figs. 10, 11). The residual 14-25nm thick lamina was less granular and the residual nuclear pores were more easily resolved. The interchromatin space was somewhat more open and fibrillar in appearance than in M HSB nuclei. More of the lightly staining amorphous material was also evident in 2M HSB nuclei. Interchromatin fibrils had diameters of 2-7nm while the interchromatin granules were about 24nm in

diameter.

Residual nuclear bodies retained their morphology and size in 2M HSB nuclei. Interestingly the residual nucleoli took on a more fibrillar appearance than in M NaCl. The extranuclear filaments apparently became aggregated for the most part in the 2M salt and bundles of these filaments often lay trapped between residual nuclei.

After final nuclease treatment (DNase with or without RNase) the residual salt-resistant nuclear matrix was obtained by centrifugation. Matrix morphology was little changed from the 2M HSB nuclei although the mean diameter decreased slightly to approximately 45% of the original *in situ* nuclear value (Table 2). Hence nuclear matrices often had a compacted appearance in the electron microscope (figs. 12, 13). Matrices from unstimulated bovine lymphocytes (fig. 11) resembled those from unstimulated mouse or human cells, in that they only contained a residual lamina surrounding a minimal fibrogranular internal network. Matrices with substantial internal fibrogranular material were only obtained from stimulated cells, presumably derived from types 2 and 3 nuclei.

The residual nuclear lamina was 20-25nm thick and was similar to the corresponding structure in 2M HSB nuclei. Intranuclear ultrastructural detail was likewise unchanged for the most part (figs. 12, 13). Residual NB were about

45% of their original diameter but were still morphologically similar to *in situ* structures. Residual nucleoli took on a highly condensed appearance in the nuclear matrix which was quite different from previous structures.

Extranuclear filaments persisted throughout the isolation procedure and these appeared to remain tightly attached to nuclear matrices (fig. 12). Also observed in the final salt- and nuclease- resistant pellet were extracted whole cells and matrix fragments (fig. 13). Residual nuclear components present in extracted but unbroken cells closely resembled nuclei in regressively stained intact lymphocytes (Setterfield *et al*, 1983). The ultrastructure of nuclei within extracted whole cells seemed to be retained very well (fig. 14). Unlike isolated nuclear matrices there remained a residual double nuclear membrane and an ordered IM. In addition, areas where chromatin had been extracted were more obvious as there was little or no collapse of residual "*in situ*" nuclei. From this observation it becomes obvious that during isolation of free nuclear matrices there occurs significant structural rearrangement of at least the internal components of nuclei. This may result simply from collapse or contraction of isolated and extracted nuclei once support by the cytoskeleton has been removed. This question is addressed further in Chapter 2.

Figure 10. Electron micrographs of 2M HSB nuclei from bovine lymphocytes.

Figure 10a. Electron micrograph of a nucleus extracted with 2M HSB. Patches of aggregated extranuclear filaments are visible (arrowheads). The residual nuclear envelope and nuclear pores (arrows) are more easily resolved than in LMB or M HSB nuclei, and the IM is more delicate in appearance. A residual NB (RNB) is visible. Bar = 0.5um.

Figure 10b. Electron micrograph of a 2M HSB nucleus. The large empty spaces and smaller amounts of filamentous IM suggest type 1 origin. Bar = 0.5um. See also Figure 11.

Figure 10c. High magnification micrograph of two adjacent 2M HSB nuclei. Many aggregated filaments occupy the extranuclear space. Residual pores are visible (arrowheads) with other details as above. Bar = 0.5um.

Figure 11. Electron micrographs of residual nuclear structures isolated from unstimulated bovine lymphocytes.

Figure 11a. LMB nucleus. Note the granular residual nuclear envelope and large open spaces within nucleus. Bar = 1.0um.

Figure 11b. M HSB nucleus contains minimal IM. Extracted cytoplasm from another cell is at left side of figure. Bar = 1.0um.

Figure 11c. 2M HSB nucleus appears similar to M HSB nucleus except open spaces are partially filled with IM. Bar = 1.0um.

Figure 11d. Nuclear matrix from a -con A lymphocyte. Minimal IM and large open spaces are intact. Bar = 1.0um.

Figure 12. Electron micrographs of nuclear matrices from stimulated bovine lymphocytes.

Figure 12a. Overview of the final nuclear matrix fraction. Intranuclear networks are extremely compact although empty spaces are visible in some matrices. Large masses of aggregated cytoplasmic filaments lie between many matrices. Bar = 1.0um.

Figure 12b. A typical contracted nuclear matrix, containing several fibrillar NB (arrowheads) and a residual nucleolus with fibrillar and granular zones. The residual NE is not well resolved and the IM appears to be composed of primarily granular structures. Bar = 0.5um.

Figure 12c. A high magnification micrograph of the nuclear matrix in Fig. 11b, showing the condensed IM. Numerous fuzzy 20-40nm granules are interspersed with fine fibrils (arrowheads) and lightly stained amorphous material (arrows). Bar = 0.25um.

Figure 13. Electron micrographs of nuclear matrices from bovine lymphocytes.

Figure 13a. Note small relative size of the nuclear matrix compared to residual NB and nucleolus, and density of IM. The matrix may have fractured near the RNB (arrowhead). The structure of the RNB is very similar to NB *in situ*; note connections to fibrogranular IM. Bar = 1.0um.

Figure 13b. Note large amounts of aggregated extranuclear filaments. Some open spaces remain within the matrix. A large RNB retains the structure observed *in situ*. Bar = 1.0um.

Figure 13c. An electron micrograph comparing an extremely contracted isolated nuclear matrix (right) and an extended *in situ* nuclear matrix within an extracted cell. Note double nuclear membrane (arrowheads), fibrogranular IM and large open spaces in the latter structure. Arrow indicates a pair of extracted centrioles. Bar = 1.0um.

Figure 14. Electron micrographs of unhomogenized bovine lymphocytes exposed to the matrix isolation procedure.

Figure 14a. Triton-extracted cell appears intact, with nuclear morphology unaffected. Arrow indicates intact mitochondrion. Bar = 1.0um. Figure 14b. A DNase-treated cell with disrupted cytoplasm (centriole; arrow). The nuclear interior has a lower electron density and chromatin appears highly condensed. Bar = 1.0um. Figure 14c. LMB-extracted lymphocyte also has disrupted cytoplasm, contains centriole (small arrow). A residual double nuclear envelope (nm) is visible in areas. Condensed chromatin has been extracted, leaving holes (large arrow). Bar = 1.0um. Figure 14d. Lymphocyte extracted with 2M HSB has appearance similar to LMB cell. Residual double nuclear membrane (arrow) and large empty spaces remain. An isolated HSB nucleus is at right. Bar = 1.0um. Figure 14e. 2M HSB cell appears similar to LMB and M HSB cells. Bar = 1.0um.

Figure 14f. An extracted lymphoblast contains an *in situ* nuclear matrix, containing residual double nuclear membrane (nm), many open internal and peripheral spaces (small arrows). A contracted isolated nuclear matrix (arrow) is at right. Bar = 1.0um.

B. PROTEIN COMPOSITION OF NUCLEAR MATRIX

1. 1-D SDS-PAGE

Proteins of bovine lymphocyte nuclei and nuclear matrices were separated by one-dimensional SDS-PAGE in order to determine if differences in polypeptide composition were evident. Gels of total nuclear proteins from unstimulated and stimulated lymphocytes were similar, with histone proteins predominating. Weaker protein bands between 40 and 80k were found, but differences in band intensities between samples were not easily resolved (not shown).

Steps consisting of DNase 1 digestion or salt extractions of nuclei each removed primarily histones and two bands between 53 and 59k. Figure 15 shows proteins remaining in DNase-digested nuclear matrices from unstimulated lymphocytes. This sample was also sorted by 2-D PAGE (below). By 1-D PAGE major proteins were resolved at 180k, 63k, 44 to 49k (5 bands), 37 to 42k (several bands) and 30 to 31k. Some differences in protein content were found in matrices from 36h ps lymphocytes (also sorted by 2-D PAGE, below). Major species in figure 16 included 170k, 130k (2 bands), 115k, 85k, 75 to 80k, 70k, 61k, 43 to 48k (several bands), 34 to 36k and 30 to 32k. More large proteins were found in active matrix, especially between 70 and 85k and at 115k.

and 130k. Some differences were visible in Figures 15 and 16, between 31 and 42k; for example, bands at 37 to 42k were stronger in -conA matrix, while a 34 to 36k component was strong in 36h ps matrix. These differences were resolved better using 2-D PAGE (see below).

The primary difference between matrix preparations was efficiency of histone extraction and, conversely, enrichment of NHP. In general, histone proteins were largely removed from residual nuclear matrices, leaving proteins of 30 to 180k.

While it is possible to separate nuclear matrix polypeptides using 1-D electrophoresis the degree of resolution available is inadequate for meaningful analysis of such complex mixtures of polypeptides. For example, it is not possible to determine whether a single band on a 1-D gel represents a single protein species, a group of charge isomers with similar MW or a number of distinct polypeptides. Furthermore, if one of the latter situations is true then it is not possible to evaluate changes in amount of, for example, one of the isomers. Hence a great deal of information could be overlooked if only 1-D gels were utilized to study nonhistone proteins.

2. Two-Dimensional Electrophoresis

In order to obtain more refined information regarding the biochemical composition of bovine lymphocyte nuclear

Figure 15 (left). One-dimensional SDS-PAGE gel of DNase 1-digested nuclear matrix from unstimulated bovine lymphocytes.

Figure 16 (right). One-dimensional SDS-PAGE gel of DNase 1-digested nuclear matrix from bovine lymphocytes 36h ps with conA.

Mr of standard proteins (right) in kd.

matrices before and during conA stimulation the 2-D PAGE procedure described by Peters and Comings (1980) was followed. Denatured polypeptides were first separated in isofocussing gels on the basis of net molecular charge, or pI, and then further sorted in SDS slab gels on the basis of MW. Within this section the terms protein and polypeptide will be used interchangeably in describing the denatured polypeptides sorted by PAGE.

Two-dimensional PAGE gels run to pI equilibrium in the first dimension were found to resolve proteins of MW 20-90k, with some exceptions. Polypeptides larger than 90k usually streaked in the first dimension gels; that is, distinct charge isomers were not resolved. Furthermore the MW scale above 90k was very compressed and MW estimates were not always reliable. Isoelectric points (pI) of about 5.0 to slightly less than 8 (about 7.7) were obtained.

Nonequilibrium pH gradient electrophoresis (NEPHGE or NE) gels were generated using different ampholyte mixtures and electrophoresis conditions (see Materials and Methods). In these gels the acidic scale was compressed and neutral to very basic (pI>8.4) polypeptides were more clearly resolved.

In some cases MW and pI values seemed to vary slightly between different matrix samples, possibly due to different electrophoresis conditions or to actual

differences in the proteins. In general, however, different runs of a particular protein sample had consistently close MW and pI values. Furthermore different matrix preparations from lymphocytes stimulated for the same time were found to have nearly identical gels. Designations of presumed similar proteins in different samples are summarized in Tables 3 and 4 (see below). The results shown are typical and reproducible.

(a) Nuclear Matrix From Unstimulated Lymphocytes

An equilibrium 2-D gel (Coomassie blue) of nuclear matrix proteins from unstimulated (-con A) bovine lymphocytes is shown in fig. 17. This gel shows a relatively simple composition with primarily acidic protein species (near A). Major components had MWs of 38, 43, 49, 52-53, 54, 58, and 68k. This "typical" arrangement of acidic components was seen in every matrix sample observed. Silver staining of the same gel as in fig. 17 revealed a number of minor and more basic polypeptides (Fig. 18; for unknown reasons the strong spots in fig. 17 remained unstained with silver). The bulk of the new spots focussed between A and C and usually appeared as single spots or small groups of evenly spaced charge isomers with similar MW. Looking at the drawing of Fig. 18 several families of presumed charge isomers were identified, including those with MW of 68A, 59C-B, 58A-S,

54C-B, 54A, 52C, 52A, 49A, 46C-B, 43A, 43B, 42C, 40C-B, 38C-B, 32B-A and 27.5B. No polypeptides larger than 68k were resolved. For reasons to be discussed below, three major families have been designated lamin B (B; 68A), vimentin (V; 58 A-S) and actin (a; 43A).

A silver-stained gel of matrix proteins of unstimulated cells, separated by NEPHGE (fig. 19), shows a similar overall profile to the equilibrium gel, but with a greater content of basic proteins. The arrangement of acidic proteins near A was as in figure 17. Lamin B (68A and 68B-A) was the major species observed. The vimentin and actin spots were also strong. Other prominent polypeptides were found at 57B (3 spots), 49A-S, 490-Hn, 42Hn-C (several overlapped spots), 40-410-Hn (5 isomers), 39C-B (several spots), 37Hn and 35C. Several minor polypeptides with MW 29-67k were also noted (see Table 3).

Unlike the equilibrium gel of -conA matrix, some proteins larger than 68k were stained in fig. 19. Four strong acidic species (75A, 81A, 92S, 105S) were seen. Two groups of basic charge isomers (77 and 81Hn-C) were also resolved in NEPHGE gels. A number of acidic and basic polypeptides larger than 80k appeared as streaks, due to an inability to focus completely in the first dimension.

Figure 17. A 2-D equilibrium gel of nuclear matrix from unstimulated bovine lymphocytes (stained with Coomassie blue).

A. Photograph of an equilibrium gel showing simple composition of -con A nuclear matrix. β - lamin B; V - vimentin; a - actin. Molecular weight standards (right), in kd. Isoelectric point (pI) standards (above, below), as in text.

B. Overlay diagram of the gel in A. Standards as above. Numbers on diagram indicate MW of proteins.

8.1 7.4 6.7 6.1 5.5
A

B
V
-68
-43

-29

-21

P 8.1
B
C 7.4
B 6.7
A 6.1
S 5.5

58
58
53
-68
-43
38

-29

-21

Figure 18. A 2-D equilibrium gel of nuclear matrix from unstimulated bovine lymphocytes (stained with silver).

A. Photograph of gel in Fig. 17 stained with silver. Some anomalous background stain is present. The most abundant proteins are not stained (eg. lamin B, vimentin, actin, others).

B. Overlay diagram of the gel in A. Putative families of protein charge isomers are circled with dotted lines. Open circle indicate proteins unstained by silver.

Figure 19. 2-D nonequilibrium gel of nuclear matrix from unstimulated bovine lymphocytes (stained with silver).

A. Photograph of a gel of nuclear matrix proteins from -con A cells. Basic polypeptides ($pI > 7$) are resolved more clearly in this gel (compare to Fig. 18). RNP indicates the area where hnRNP core proteins are sorted. Hn indicates an internal pI standard corresponding to the most abundant hnRNP core protein (Figs. 24, 25). Other labels and standards are as in Figs. 17 to 22.

B. Overlay diagram of the gel in A. Some families of basic proteins are circled (compare to Fig. 18). Lamin B (68A) is abundant in this sample. Numbers on diagram indicate MW of proteins. Standards as above.

(b) Matrix of Lymphocytes 18h ps with Con A

A nuclear matrix sample was isolated at 18h ps with con A. At this intermediate (G1) period RNA transcription occurs but DNA replication is not observed (Butterfield et al, 1983). The type 2 nuclear morphotype is prevalent at this stage, with some type 3 lymphoblasts also present, but numerous unstimulated type 1 cells are also present (Table 1).

The Coomassie blue-stained gel in figure 20 shows a pattern very similar to that of -conA matrix (fig. 17). The major species were grouped in the acidic portion of the gel between A and S. Lamin B (68k) consisted of 5 isomers. There were three presumed vimentin spots (V; 58A-S) and 2-3 actin isomers (a; 43.5A). The strong 38k polypeptide was also prominent at 18h p.s. Other strongly stained spots were found at 54A-S and 42B. Several minor species were interspersed between 27 and 57k and C to S.

Silver staining of the same gel intensified some of these weakly stained spots and made many others visible (fig. 21). As in figure 18 some of the strong constituents in the 18h gel stained with Coomassie did not stain with silver (eg. B, V, 54A-S and a). It was apparent that the nuclear matrix from the 18h +con A sample while containing a similar pattern of acidic proteins was different from that of unstimulated lymphocytes (see Table 3). There were more proteins.

larger than 58k than in the earlier sample. For example, acidic species of 87k and larger (near A), 81B, 85C and a family of 5 charge isomers at 75C. Species at 65B and 65C were new, as was a family of 4 spots at 63C-B. Novel groups of 3 isomers each were located at 52B-A and 45C-B.

Few new proteins were observed in the quadrants bounded by C and A, 29-43.5k. Instead it appeared that minor components or small groups present in the -conA sample were represented by (larger) groups of spots. Some examples were noted at 43B, 41B-A, 40-42C, 38A and 34A.

Perhaps of significance was the reduction in intensity or shifting position of some protein spots at 18h. The group at 27C-B seemed to be shifted slightly to the basic end of the gel. Conversely the groups at 40B and 34A seemed relocated to the acidic end of the gel. The intense family of 5 charge isomers at 32B-A in the -conA sample (fig. 18) was quite diminished at 18h, with only the 2 weaker spots represented. It is unknown whether a new, weak and fairly acidic spot (32S) at 18h was related to this group or not.

Some novel, smaller proteins appeared in the 18h matrix. Several minor species of pI 7.0 or greater at 35-40k were observed. A small group at 41A-S was also noted. The unknown polypeptide at 38A-S seemed to be present at a much greater relative concentration than in the -conA sample.

Resolution of the more basic polypeptides in the 18h nuclear matrix was accomplished by NEPHGE (fig. 22). Some larger (over 105k) proteins were represented by streaks since they did not focus in the first dimension. As expected there were several similarities to the 18h equilibrium gel, particularly in the compressed acidic portions. A few larger polypeptides (eg. 105A, 83A, 81B) were common. However there were differences in the high MW parts of the equilibrium and NE gels (compare Tables 3 and 4). In the NEPHGE gel minor species were found at 69C-B, 730-Hn and 68 O-Hn; the group of proteins at 68, 70, and 78Hn-C, each contained a family of 4-6 evenly spaced spots. The isoelectric points of the 4 most basic charge isomers in each family were almost identical. In the equilibrium gel only one family (75C) was resolved in this region.

The relative intensities of spots in the quadrants bounded by A and C (43-68k) appeared to be decreased in the NE gel although the overall patterns remained similar. In this intermediate range a few more basic polypeptides were detected using NEPHGE; for example, a double spot at 65Hn, a triplet at 56Hn and larger families at 59 O-Hn and 54 O-Hn. Two spots at 61 O-Hn and 48 O-Hn were also observed in the nonequilibrium gel.

Below 43k novel very basic proteins were again resolved by the NEPHGE system. A group of 5 spots at

38-390-Hn formed one family. Two other large families were found at 36Hn-C and 34 0-C. A small group was also observed at 29 0-Hn.

Comparing the 18h +con A NEPHGE gel (fig. 22) to the respective -conA gel (fig. 19) illustrates the numerous similarities and apparent changes after conA stimulation. Some large species were common to both fractions (eg. 105A, 83A, 81B), while only the 18h matrix sample contained 69C-B, 73 0-Hn and 68 0-Hn. In addition the group at 68-78Hn-C was much more complex at 18h. The lamin B spots were much more prominent in the -conA matrix.

Intermediate size polypeptides common to the -conA and 18h matrices included 65Hn, 54 0-Hn and 48 0-Hn. Only the stimulated matrix was found to contain 61 0-Hn and the families at 59 0-Hn and 56Hn. Several differences were observed among small polypeptides. The strong spot at 39C-B in the -conA matrix was virtually non-existent at 18h. Three large families of basic polypeptides were very strong at 18h, but were negligible or absent from the -conA matrix (36Hn-C, 34 0-C, 29 0-Hn). The family of five charge isomers at 38-39 0-Hn in the 18h matrix likely corresponded to 40-41Hn in the -conA matrix. The relative intensities, positions and arrangements of spots were virtually identical in gels from the two times.

Figure 20. 2-D equilibrium gel of nuclear matrix from bovine lymphocytes 18h ps with con A (stained with Coomassie blue).

A. Photograph of gel of nuclear matrix proteins from 18h stimulated population of cells. Coomassie staining resolves a simple composition for this sample. Labels and standards as in Fig. 17.

B. Overlay diagram of the gel in A, with designations as noted in Fig. 18.

A 8.1 7.4 6.7 6.1 5.5

B -68
V
-43

-20
-21
P 8.1 C 7.4 B 6.7 A 6.1 S 5.5

B
57
-68
-43
-38
-32.5
-31
-27
-21

Figure 21. A 2-D equilibrium gel of nuclear matrix from bovine lymphocytes 18h ps with con A (stained with silver).

A. Photograph of the gel in Fig. 18 stained with silver. Silver staining allows resolution of the complexity of this sample. As in Fig. 18 some anomalous background stain is present and the most abundant proteins are not stained with silver (lamin B, vimentin, actin, others). Labels and standards as in Fig. 17.

B. Overlay diagram of the gel in A. Numerous protein families are circled with dotted lines. Designations are as in Fig. 18.

8.1

7.4

6.7

6.1

5.5

A

-68

-43

-29

321

P

8.1

C

7.4

B

6.7

A

6.1

S

5.5

B

-87

75

68

-68

65
58
54

63

58
54

-43

40
36

34

38

32.5

-29

27

28

-21

Figure 22. 2-D nonequilibrium gel of nuclear matrix from bovine lymphocytes 18h ps with con A (stained with silver).

A. Photograph of a gel of nuclear matrix proteins from 18h ps con A cells. Families of basic proteins greater than 68k are abundant, as are several putative RNP proteins (area near RNP). Labels and standards as in Fig. 19.

B. Overlay diagram of the gel in A. Note the complexity of the families of high pI proteins, particularly the RNP proteins (340-C, 360-C, etc.). Standards as above.

A

B

In summary, it can be said that with the appearance of the types 2 and 3 morphotypes by 18h p.s. several alterations in the nuclear matrix proteins were seen. Aside from a few decreased or absent spots the 18h sample contained several novel protein species, while some consistent species seemed to have higher numbers of charge isomers.

(c) Matrix of Lymphocytes 36h ps with Con A

By 36h after addition of conA to bovine lymphocytes a large proportion of cells had type 3 morphology (Table 1); that is, the cells and nuclei were quite enlarged and the nuclei were structurally reorganized (Setterfield et al, 1983). At this time many of these lymphoblasts are undergoing DNA replication. Therefore a sample of nuclear matrix from 36h post conA lymphocytes was selected to represent the "activated" state. A small proportion of type 1 cells remain in the sample, presumably contributing an unstimulated composition to the analysis.

An equilibrium gel of 36h +con A matrix stained with Coomassie blue and silver is shown in figs. 23 and 24, respectively. As observed in 18h gels, several proteins larger than 68k were present. Groups of charge isomers at 82C-B, 78B and 77C were present at both 18h and 36h p.s. The 36h sample showed greater complexity and intensity in these areas and showed numerous new spots (eg. 86B, 76C-B,

72A, 68C-B; fig. 24). The group at 76C-B in fig. 23 may be lamin A, as will be discussed later.

Many of the proteins in the intermediate size zone (43-68k) which first appeared at 18h ps remained at 36h ps, usually with many more spots per group (see Table 3). Polypeptides conserved in all 3 samples are listed in Table 3 and include lamin B (68A), vimentin (58A), 57C-B, 54Hn-C, 54C-B, 52.5A-S, 52C-B, 51B-A, 47C-B, 49A, 47A, 45C-B, actin (44A) and 43B-A. In general each polypeptide group was more complex at later stimulation times. Furthermore, many of these spots were relatively more intense at 36h than at 18h ps. In addition to these groups which increased in complexity and intensity several intermediate size protein families unique to the 36h nuclear matrix were found, at 68B-A, 65C-B, 60C, 57Hn-C, 49B-A and 43.5C-B. A seventh family (63C) consisting of 5 spots may have been lamin C.

The same trends noted above can be extended to the smaller polypeptides between 29 and 43k. Groups of proteins found in all 3 samples of matrix included 41C-B, 40C, 39C-B, 38A-S, 37B-A, 34B-A and 32A (Table 3). The protein designated 38A-S increased quite noticeably in intensity from 0-18h and from 18-36h ps. The protein was also more basic as stimulation progressed. Again, many of the spots at 36h were more intense than at 0 or 18h (eg. 40C, 39C-B). An exception to this trend was the group at

32C. In the -conA matrix (figs. 17,18) the five spots in this area were very strong but by 18h (figs. 20,21) only two small spots were seen; these were even less intense by 36h ps. In addition the doublet at 40C-B which was present in the -conA matrix but absent from the 18h matrix, did not appear at 36h either. Alternatively seven groups of polypeptides were found only in the activated (18h, 36h) nuclear matrices (near 36C-B, 35C-B, 34D, 33A, 32A-S, 30.5A and 30B-A). The most striking observation here was amplification in the first two groups at 36h ps. The family at 36C-B consisted of 12 spots at 36h (vs. 2 spots at 18h) while 35C-B contained 6 spots at 36h vs. 2 spots at 18h. Some small polypeptides present only in the 36h nuclear matrix included groups at 39B-A, 38C, 36A-S and 34A-S.

A fourth group of proteins, smaller than 29k, showed similar responses. One isomer family at 27.5B-A was in all 3 matrix samples while families at 28B-A, 27C-B and 26S were only found in the two stimulated samples.

Several minor spots, smaller than 25k, were only observed in 36h ps matrix. A doublet near 33B was observed in the -conA and 36h ps matrices, but was undetected at 18h.

Separation of the proteins in the 36h +con A nuclear matrix by NEPHGE (fig. 25) revealed further details of composition which were compared to probable corresponding polypeptides in -conA and 18h ps NE gels (Table 4). Above

68k the major families were similar for all 3 matrix samples (eg. 86A-S, 81Hn-C, 79Hn-C, 76A). The basic groups at 81Hn-C and 79Hn-C consisted of 5 evenly-spaced spots each; the charge isomers in these families had identical pI in each sample. No significant differences among the 3 samples were noted in basic regions of gels.

Notable deviations in protein composition were found in the intermediate size range (43.5-68k). Of nearly 18 families of proteins only 6 minor ones were found in all 3 matrix preparations (ie. 65Hn-C, 55B, 50C-B, 49B-A, 48 0-Hn, 44Hn-C). A few groups were absent only from the -conA sample (ie. 68Hn-C, 65C(laminC?), 65B, 57Hn-C, 43.5Hn). Proteins unique to the 36h matrix sample were 54C and 47Hn doublets.

Among smaller polypeptides few obvious additions were noted at 36h ps, as compared to 18h ps. Two notable differences occurred at 37.5-39C-A and 32Hn. In the former case one (or two close) group(s) of 11 charge isomers present at 36h was missing completely at 18h ps. A relatively strong unfocussed streak was found in this position in the -conA matrix gel (fig. 19). The spot at 32Hn where a triplet was found -conA was also not present in the 18h nuclear matrix. Some of the polypeptide families found at 18h, which were weak or absent from the -conA sample, were markedly expanded at 36h ps. The group at 40.5 and 41.5B-A is an example. Another is the

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elaboration of the groups at 34 O-C and 36 O-C. Also two closely migrating families of 5 spots each (39 and 39.5 O-Hn) were probably related to the single pentamer groups at 38-39 O-Hn (18h; fig. 22) and 40-41 O-Hn (-conA; fig. 19).

Figure 23. A 2-D equilibrium gel of nuclear matrix from bovine lymphocytes 36h ps with con A (stained with Coomassie blue).

A. Photograph of a gel of nuclear matrix proteins. Coomassie blue stains a large number of proteins (compare to Figs. 17, 19). A and C indicate lamins A and C, respectively. Other labels, standards as in Fig. 17.

B. Overlay diagram of the gel in A. Numbers indicate MW of protein charge isomers.

8.1 7.4 6.7 6.1 5.5

A

-C -A

B

-68

-43

-29

-21

P 8.1 C 7.4 B 6.7 A 6.1 S 5.5

B

77 72

72 68

-68

57

62

58

-43

54 51

52

40

38

36

36

34

-29

-21

Figure 24. A 2-D equilibrium gel of nuclear matrix from bovine lymphocytes 36h ps with con. A (stained with silver).

A. Photograph of a gel of nuclear matrix proteins, in which silver stain resolves a very large number of proteins. As in previous samples the most abundant proteins are unstained. Labels and standards as in Fig. 17. B. Overlay diagram of the gel in A, showing the large groups of charge isomers. Open circles indicate proteins which were not stained by silver. Numbers indicate MW of proteins (in kd). Standards as above.

Figure 25. 2-D nonequilibrium gel of nuclear matrix from bovine lymphocytes 36h ps with con A (stained with silver).

A. Photograph of a gel of nuclear matrix proteins from 36h ps con A cells. This gel shows similarities to the 18h gel in Fig. 22. C indicates lamin C. Other labels and standards as in Figs. 17, 19.

B. Overlay diagram of the gel in A. Compare to 18h sample in Fig. 22. Labels and standards as in Fig. 19.

TABLE 3 - Polypeptides in Equilibrium 2-D Gels

Time After Con A Addition			
0h	18h	36h	
----	----	86B	(3)
----	85C (1)	82C-B	(5)
----	81B (1)	78B	(6)
----	75C (5)	77C	(2)
----	----	76C-B	(6)
----	----	72A	(4)
68A (3)	68A (5)	68A	(4+)
----	----	68B-A	(5)
----	63C-B (4)	63C-B	(2)
----	----	63C	(5)
----	61B-A (2)	62B-A	(2)
----	----	60C	(2)
----	57B-A (2)	59B-A	(9)
58A-S (2)	58A-S (4)	58A	(3+)
----	----	57Hn-C	(3)
58C-B (2)	58C-B (4)	57C-B	(3)
54Hn-C (2)	----	55Hn-C	(3)
54C-B (3)	54C-B (4)	54C-B	(4)
52A (1)	54A-S (2)	52.5A-S	(2)
50A (1)	50B-A (3)	51B-A	(4)

Table 3 (continued):

-----	-----	49B-A (7)
49A (2)	48A (1)	47A, 49A
47C-B (1)	47C-B (2)	47C-B (6)
-----	46A (2)	46A (2)
46C-B (3)	45B (1)	45C-B (7)
44A (4)	44A (4)	44A (4)
-----	-----	43.5C-B (5)
43B (2)	43B-A (4)	43B-A (7)
41.5B-A (1)	41B (4)	41C-B (4)
40C-B (2)	-----	-----
38C-B (6)	40B (2)	39C-B (6)
-----	-----	38C (1)
38A-S (+)	38A-S (++)	38A-S (+++)
37B-A (3)	37B-A (2)	37B-A (5)
-----	36C (2)	36C-B (12)
-----	-----	36A-S (4)
-----	35C-B (2)	35C-B (6)
-----	34B (2)	34B (1)
34B-A (1)	34B-A (1)	34B-A (1)
-----	-----	34A-S (2)
32.5B (1)	-----	33C-B (1)
-----	34A (2)	33A (2)
32B-A (5)	32.5A (2)	32A (2)

Table 3 (continued):

----	32S (1)	32A-S (2)
----	31A (1)	30.5A (2)
----	30B-A (1)	30B-A (1)
----	28B-A (2)	28B-A (3)
27.5B (4)	27.5B-A(2)	27.5B-A(3)
----	27C-B (4)	27C-B (6)
----	26S (2)	26S (2)

1. For key to nomenclature see Results section.
2. Numbers in brackets refer to number of isomers.
3. A '+' in brackets refers to relative intensity.

TABLE 4 - Polypeptides in Nonequilibrium 2-D Gels

Time After Con A Addition		
0h	18h	36h
81A-S (1)	83A (1)	86A-S (1)
81Hn-C (4)	78Hn-C (6)	81Hn-C (4-5)
77Hn-C (3)	70Hn-C (5)	79Hn-C (5)
75A (*)	72A (*)	76A (4)
----	69C-B (1)	----
----	69Hn-C (4)	68Hn-C (2)
67Hn (1)	65Hn (2)	67Hn (1)
----	61C-B (*)	65C (3)
67 O-Hn(1)	65Hn (2)	65Hn-C (1)
----	61 O-Hn(1)	----
----	60B (1)	65B (3)
----	59 O-Hn(6)	57 O-Hn(3)
----	56Hn (3)	57Hn-C (1)
57B (3)	55B (1)	55B (2)
54Hn (*)	54 O-Hn(5)	54Hn (*)
----	----	54C (2)
51 Hn-C(*)	52C (5)	50C (5)
47B-A (3)	48B (1)	49B-A (3)
49 O-Hn(1)	48 O-Hn(1)	48 O-Hn(3)
----	----	47Hn (2-3)

Table 4 (continued):

44Hn-C (3)	44C (3)	44Hn-C (4)
----	43.5Hn (1)	43.5Hn (2)
42Hn-C (*)	42Hn-C (4)	41Hn-C (4)
41B-A (2)	41B (2)	41B-A (2)
----	40B (1)	40B-A (5)
40 O-Hn(5)	39 O-Hn(5)	39.5 O-Hn(5)
----	---/---	39 O-Hn (5)
39C-A (*)	----	39C-A (1)
37Hn (4)	36 O-C (6)	36 O-C (6)
37C-B (1)	36.5B (3)	36C-B (2)
-----	34 O-C (8)	34 O-C (12)
----	34B-A (1)	33B-A (1)
35C (2)	33C (1)	33C (3)
32Hn-C (3)	----	32Hn (1)
----	29 O-Hn(3)	29 O-Hn(1)
----	26.5B, 25.5B	27B (1)
20Hn-C (1)	21Hn-C (1)	20Hn-C (1)

1. For key to nomenclature see Results section.
2. Numbers in brackets refer to number of isomers.
3. A '*' in brackets indicates a streak of stain.

DISCUSSION

A. Morphological Changes in Stimulated Cells

In this study the morphological changes observed in bovine retropharyngeal lymphocytes in response to con A resembled those in human PBLs (Bladen, 1980) and mouse splenocytes (Setterfield et al., 1983). Nuclear volume increased several-fold and large condensed chromatin clumps near nuclear membranes in type 1 cells became smaller, more numerous and distributed throughout the nuclear interior in type 3 cells. The increase in nuclear volume appeared to be primarily due to a marked increase in the amount of interchromatinic material (IM). In con A-stimulated human PBL there is a 10-fold increase in this component during mitogenesis. These changes occur as transcription and protein synthesis are stimulated, prior to DNA replication, and are insensitive to inhibitors of DNA synthesis (Setterfield et al., 1983). Transition of *in situ* nuclear morphology from type 1 to type 3 likely represents a progression from G0 to G1 in the cell cycle (Setterfield et al., 1985).

Nuclear swelling and condensed chromatin disaggregation seem to be general responses to enhanced transcriptional activity or preparation for DNA replication; for example, after nuclear transplantation (Graham et al., 1966) or in avian erythrocyte nuclei.

reactivated in heterokaryons (Lafond et al, 1983). The opposite sequence of events occurs during maturation of avian erythrocyte nuclei as interchromatinic material disappears (Dardick and Setterfield, 1976). Clearly nuclear size and internal organization seem to be highly dependent on the elaboration of IM, as well perhaps as the surface area of the pore complex-lamina. The latter must change during nuclear enlargement to maintain the dimensions observed in smaller nuclei.

The results of the present study are similar to those of Lafond et al (1983) where inactive and active chick erythrocyte nuclei were compared. The nuclear matrix was reportedly not required for nuclear structure or chromatin organization in inactive nuclei. Such nuclei are from cells which are terminally differentiated and have more extreme chromatin aggregation and metabolic shutdown than lymphocytes. Reactivation of erythrocyte nuclei by fusion with cells or cytoplasts led to RNA synthesis, DNA synthesis and nuclear swelling, as found for con A stimulated lymphocytes. A typical nuclear matrix formed within 16h, with more matrix in larger nuclei. Lafond et al (1983) concluded that the intranuclear matrix was a transcriptional network present only in active nuclei. Unlike lymphocytes (Setterfield et al, 1983a) addition of cycloheximide or alpha-amanitin did not block the morphological changes in chick nuclei (Woodcock et

al, 1984), indicating use of pre-existing nuclear proteins from the cytoplasm, rather than *de novo* synthesis.

B. Isolation of Lymphocyte Nuclear Matrix

The bulk of evidence in the literature to date indicates that the nuclear matrix has a vital functional role in ordering many nuclear events (see Agutter, 1985). As a working hypothesis in this discussion I propose that the nuclear matrix is a genuine nuclear component which comprises the pore complex-lamina and interchromatinic material (isolated in variously modified forms) and contains the apparatus required for a variety of nuclear metabolic processes. As shown in past studies the *in situ* IM is morphologically similar to that in isolated nuclear matrix (eg. Berezney and Coffey, 1977; Setterfield et al, 1983a, Literature Review). Association of several metabolic processes with nuclear matrix have been proposed including, among others, DNA replication (Berezney and Buchholtz, 1981a, b), hnRNA synthesis (Mirkovitch et al, 1984; Jackson and Cook, 1985b), hnRNA processing (Mariman et al, 1982) and RNP translocation to the cytoplasm (Agutter, 1984; 1985). It must be acknowledged that the nuclear matrix is a complex biochemical fraction which may or may not be an accurate representation of the actual *in vivo* nuclear structure. However, a fibrillar *in situ* nuclear matrix has been visualized in EM sections of

intact liver nuclei after clearing of masking chromatin and RNP by α -amanitin (Brasch, 1982) and a similar structure has been isolated intact by Agutter (1985).

Nuclei isolated from bovine lymphocytes using Triton X-100, retained their shape and morphology. Slight compaction of chromatin may have been due to the presence of Mg^{++} ions, as the concentration of divalent cations affects chromatin ultrastructure in isolated chicken liver nuclei (Brasch, 1982). DNase 1 digestion of isolated nuclei caused no significant change in nuclear appearance, suggesting a non-chromatin composition for the fine 2-6nm interchromatinic fibrils, and the interchromatin granules. Extraction of nuclei by LMB caused significant changes in nuclear morphology as histones, and presumably DNA (Berezney and Coffey, 1977), were solubilized. Condensed chromatin appeared to have been extracted as large open spaces were visible in many nuclei in positions similar to those occupied by heterochromatin *in situ*. This was most obvious in extracted -con A nuclei. The intranuclear network resolved after LMB extraction resembled the *in situ* intranuclear matrix (IM). Extraction of LMB nuclei by a gradual increase in NaCl concentration removed more chromatin but resulted in few significant morphological changes. Large open spaces were observed in some nuclei but not in others. Loss of open intranuclear spaces may have been due to compaction or reorganization of nuclei in

high salt., Isolated Tetrahymena nuclear matrices reversibly contract depending on concentration of divalent cations. Removal of chromatin may allow this to occur in bovine lymphocyte matrices. Other factors which may explain the contraction of residual nuclei will be discussed below.

A final DNase (or DNase 1-RNase A) digestion resulted in only minor morphological changes. A small reduction in nuclear matrix (NM) diameter could have resulted from extraction of residual chromatin and RNA/P (Berezney and Coffey, 1977), giving bovine lymphocyte NM a more compacted appearance in the EM. Nuclear matrices prepared with or without RNase were indistinguishable by EM, suggesting that residual RNA has little structural role in bovine lymphocyte matrices unless it remains in a nuclease-resistant RNP. Some groups (eg. Bouvier *et al.*, 1985a,b; Long and Schrier, 1983; Kaufmann and Shaper, 1984) suggest that the intranuclear matrix is an artifactual aggregation of transcriptional complexes.

However this proposal has been disputed by other workers (eg. van Eekelen and van Venrooij, 1981; Jackson and Cook, 1985). It remains to be unequivocally demonstrated that RNA/P play crucial structural roles in the nuclear matrix.

It is not known whether residual proteins and nucleic acids were aggregated during high-salt extraction.

However, the largest change in morphology observed here occurred in LMB. The subsequent gradual increases in NaCl concentration used likely reduced aggregation artifacts which may have resulted from rapid changes in ionic strength used in other studies. While the residual nuclear diameter decreased in HSB, the morphology of IM components was unchanged compared to LMB nuclei. Some aggregation or stabilization of fibrogranular material and residual nucleoli may have resulted from inclusion of Mg^{++} ions in extraction buffers used in this study (Brasch, 1982; Long and Ochs, 1983).

The intranuclear matrix of bovine lymphocytes was very sensitive to endogenous proteases, confirming the importance of proteins in matrix stability. Failure to include at least PMSF in buffers resulted in loss of or damage to the IM. NaTT was included in buffers as a sulphhydryl protease inhibitor (Berezney, 1979). Kaufmann and Shaper (1984) have shown that in rat liver NM this agent induces intermolecular bonds which stabilize the IM.

This data has been disputed by others (eg. van Eekelen and van Venrooij, 1981; Abulafia et al, 1984) who used sulphhydryl-blocking reagents and isolated identical matrices. The potential for disulphide formation in lymphocyte nuclear matrix was not examined in the experiments reported here but it is possible that, after removal of chromatin from lymphocyte nuclei, such cross-

links could have formed to stabilize the IM. Kaufmann and Shaper (1984) suggested that the data of Lafond et al. (1983) illustrated the stabilization of a salt- and nuclease-resistant intranuclear network in nuclei from metabolically active cells by NaTT. However, Lafond et al. (1983) showed that NaTT was not responsible for generating the intranuclear matrix in their studies.

Nuclear matrices from unstimulated lymphocytes resembled pore complex-lamina with minimal IM, consistent with the appearance of *in situ* nuclei. On the other hand, matrices isolated from con A-stimulated cells contained an extensive intranuclear network of salt- and nuclease-resistant 2-6nm fibrils, 10-25nm granules and an amorphous, lightly-staining component. Although contracted, the overall dimensions and arrangement of this material resembled the elaborate *in situ* IM. It is assumed that these structures are nonchromatin NHP components of lymphocyte nuclei since they resisted extraction, were present in samples which showed little or no histone protein (or DNA; see Hoechst stain in Chapter 2) and resembled RNP stained by EDTA-regressive uranyl in the EM (Setterfield et al., 1983a). The markedly different morphologies of quiescent vs. active lymphocyte nuclei were retained in isolated nuclear matrices and intermediate nuclear fractions. This is consistent with the hypothesis that the NM is derived from the PCL and IM

components of *in situ* nuclei.

Nuclear matrices from con A-stimulated bovine lymphocytes appeared different from IM of mouse lymphocytes. Whereas bovine matrices were often compacted, mouse matrices had an extended morphology like the *in situ* IM, with large open spaces where condensed chromatin had been extracted (Setterfield et al, 1983). Although unlikely, this may indicate a species-dependent reaction of isolated NM to high ionic strength. In the present study some lymphocytes failed to lyse in detergent and the resultant cells which had been exposed to NM isolation contained residual nuclei with extended IM and open spaces resembling *in vitro* LMB nuclei. Nuclear shape and volume are retained when intact HeLa cells are passed through a NM extraction procedure (Staufenbiel and Deppert, 1984). This may imply an active role for the residual cytoskeleton in maintaining gross morphology (and volume) of NM, perhaps mediated through intermediate filaments (IF) and/or substrate attachment. Condensed chromatin in Triton or *in situ* nuclei could keep the spaces observed in LMB nuclei open by passive action. After chromatin removal addition of HSB may cause the surrounding interchromatinic material to contract and fill these spaces. In many mouse lymphocyte nuclear matrices (Hall, 1982) some residual cytoplasm remained and may have prevented collapse of spaces in HSB nuclei.

C. Cytomatrix Components in Nuclear Matrix

An interesting aspect of bovine lymphocyte NM isolation was the persistence of visible cytoplasmic filaments attached to the residual nuclear envelope throughout the extraction series. Their size, resistance to DNase 1 and insolubility in LMB and HSB suggested these were intermediate filaments. Antisera against vimentin stained Triton nuclei, NM, and intermediate fractions and gave a bright perinuclear filamentous pattern (M. Paulin-Levasseur, personal communication). This is consistent with the results of Staufenbiel and Deppert (1982) who found IF tightly attached to the peripheries of isolated HeLa nuclei. An intimate association has been proposed between IF and the nuclear periphery in extracted cultured cells (Fey et al, 1984). Vimentin (53-57k) and cytokeratins (44-70k) are often major components of nuclear matrices prepared from cultured cells when polypeptides are separated by PAGE. In this regard the 1-D PAGE results in this study are similar to those with mouse lymphocytes (Hall, 1982), unstimulated bovine lymphocytes (Nakayasu and Ueda, 1981, 1983) and HeLa cells (Staufenbiel and Deppert, 1983). In those systems IF proteins seemed to account for much of the 45-60k protein complement of the matrix preparations. In this study a 50-57k doublet, which may have been IF protein, was solubilized in nearly every extraction step, perhaps due

to physical disruption of the cytofilaments. Putative IF proteins were identified in 2-D gels of NM from bovine lymphocytes. Vimentin migrates to 53-57k (pI 5.7) in gels of CHO nuclear matrix (Peters et al, 1982) and in unstimulated bovine matrix (Nakayasu and Ueda, 1983).

Abundant cytoskeletal proteins have also been identified in gels of *in situ* HeLa nuclear matrices (Staufenbiel and Deppert, 1983). In this study a spot at 53-59A was presumed to be vimentin. Due to uncertainty regarding the cytokeratin complement of bovine lymphocytes analysis of acidic to neutral polypeptides between 44-70k is complicated (see also Staufenbiel and Deppert, 1983). For example, groups of polypeptides at 47-48k (near actin) and 50-51B may be lymphocyte keratins. Immunoblotting with a variety of anti-keratin sera would allow identification of these cytoskeletal proteins. Actin (43.5k) has been identified in NM from unstimulated bovine lymphocytes by Nakayasu and Ueda (1983). It was used in this study as a 2-D gel standard (see Materials and Methods). In this

study 3-4 actin isomers were resolved in NM from lymphocytes with or without con A stimulation, although actin was relatively more concentrated in -con A matrices. Antisera against actin stained the NM periphery strongly, similar to the reaction with anti-IF sera (M. Paulin-Levasseur, personal communication). On the other hand, Nakayasu and Ueda (1983, 1984, 1985) propose that F-actin

is present within NM from unstimulated bovine lymphocytes or mouse leukemia cells as a constituent of the IM. It is presently not clear what the significance of a tight nucleoskeleton- cytoskeleton association might be (Capco et al, 1982; 1984; Fey et al, 1984) or whether cytomatrix proteins have structural or functional roles within the nucleus or NM.

4.3. Nuclear Bodies in Lymphocyte Nuclear Matrix

Large nuclear bodies, more prevalent in types 2 and 3 nuclei than type 1 nuclei, were different in detail from mouse lymphocyte NB (Chaly et al, 1983a). Numerous fibrils radiated from the fibrous shells of these structures, crossed the surrounding clear halos, and made contacts with fibrogranular components of *in situ* nuclei. This intimate association, easily resolved after LMB extraction, was maintained throughout the matrix isolation procedure. Dense fibrils located in NB shells were the same diameter as those in the NM. Nuclear body morphology was unaffected by matrix isolation; DNase digestion and HSB extraction caused volume reductions comparable to those for matrix but no further changes were observed. The molecular composition and functions of NB are not known (Dupuy-Coin et al, 1972; Chaly et al, 1983b; Radoux et al, 1984). Their increased presence in pathological or hyperactive tissues, including mitogen-stimulated

lymphocytes, suggests some association with nuclear metabolism; for example, DNA replication or RNA synthesis, processing and transport. Radoux *et al* (1984) implied a relationship between NB and nuclear matrix in LPS-stimulated mouse lymphocytes. The tight association described here between bovine lymphocyte NB and NM is consistent with this. The apparently similar structural elements, resistance to extraction and high salt contractility of NB and NM indicate potential structural and/or functional relationships. Nuclear bodies may represent specialized domains of nuclear matrices associated with particular genes, analogous to nucleoli and rDNA. Alternatively they could be involved in mobilization of RNP stored before con A stimulation as NB are not labelled with radioactive precursors during mitogenesis (Chaly *et al*, 1983b). The greater abundance and structural complexity of NB in stimulated lymphocytes (Chaly *et al*, 1983a,b; Valkov and Moyne, 1974; this study) also suggest a role in nuclear metabolic processes which are activated by lectin mitogenesis. An unequivocal involvement of NB in nuclear matrix-associated DNA and/or RNA metabolism has yet to be shown.

E. Electrophoresis of Lymphocyte Matrix Proteins

(i) 1-D PAGE of Nuclear Matrix

One-dimensional PAGE of material solubilized during matrix isolation showed that primarily histones (and likely IF) were extracted by DNase 1, LMB and HSB. In general the residual nuclear matrices from unstimulated or con A-stimulated bovine lymphocytes consisted of species between 30k and 80k. Similar results were obtained by Hall (1982) for mouse splenocytes and by Nakayasu and Ueda (1981) for unstimulated bovine lymphocytes. Major proteins had MW 44k, 53k and 68k in the latter study, corresponding to actin, vimentin and lamin B, respectively (Nakayasu and Ueda, 1983). The results reported here are consistent with these data as visible cytoskeletal elements coisolated with matrices. In gels of rat liver NM (Berezney and Coffey, 1977; Matsuura et al, 1981) a strong lamin triplet was resolved between 60-70k. In gels of matrix from unstimulated lymphocytes no such triplet was observed. The region between 60-80k is more complex in NM from stimulated lymphocytes, although a distinct triplet was not visible in these gels either. Better resolution of the proteins in this range was possible using 2-D PAGE (see below). Larger polypeptides (>80k) were present in greater quantities in gels of stimulated NM; their relative enrichment varied with regard to

efficiency of histone extraction.

(ii) 2-D PAGE of Matrix During Con A Stimulation

Two-dimensional PAGE was found to be essential for accurate and detailed comparisons of nuclear matrix polypeptides from lymphocytes. One-dimensional gels were insufficient for identification of new or unique proteins or for resolution of presumed NHP families. Two-dimensional gels of nuclear matrix from unstimulated bovine lymphocytes showed a simple composition which closely resembled that of rat liver nuclear lamina (Peters and Comings, 1980) and unstimulated mouse lymphocyte matrix (Setterfield *et al.*, 1983b). The simple polypeptide composition of NM from unstimulated bovine lymphocytes is consistent with the simple IM structure observed in the EM and reduced nuclear metabolism in these cells, i.e. a lamina with minimal intranuclear material. Major polypeptides in Coomassie-stained gels were lamin B (several isoforms at 68A), vimentin, actin and

unidentified species at 49-54k near actin. Other proteins resolved using silver stain were only minor components of -con A matrix. The intensity of silver stain does not necessarily correlate with relative concentration of protein (Peters *et al.*, 1982). Their identities will be discussed below.

Two-dimensional gels of NM from -con A and 18h ps

lymphocytes are similar in acidic regions. However, con A stimulation results in much greater complexity in neutral to basic regions of 18h ps NM gels. As discussed above, a number of significant structural changes occur in lymphocyte nuclei within 12-18h ps. De novo synthesis of stable nuclear proteins before 24h ps is necessary for nuclear modification and cycloheximide can block these processes (Setterfield et al, 1983a,b). NHP contents of individual mouse lymphocyte NM increase several-fold during the morphological transition at 10-18h ps (Hall, 1982). Similar data were reported by Polet (1983) using con A-stimulated human PBL. Continued synthesis of novel nuclear NHP occurs between 18-36h ps and thus it is not surprising that NM from 18h and 36h ps lymphocytes have progressively more complex compositions than NM from quiescent cells.

Furthermore, while nuclear matrices from 18h ps lymphocytes contain numerous novel polypeptides, matrices from 36h ps cells are even more complex. Some new NHP are observed but there is also a large increase in complexity of several existing protein families as more type 3 cells enter the population.

The 2-D NE gels of 18h and 36h ps nuclear matrix resemble NE gels of rat liver (Peters and Comings, 1980; Kaufmann and Shaper, 1984) and CHO (Peters et al, 1982) matrix, with several similar components. However, NM from

36h ps lymphocytes contains far more NHP species.

Apparent families of protein isomers with identical MW (but different pI) appeared in gels of NM proteins from stimulated lymphocytes. The polypeptides in each group are fairly evenly spaced, suggesting a quantum change in net molecular charge (pI). Peters and Comings (1980) stated that such groupings of polypeptides is not derived from preparative techniques. Instead the isomers result from post-translational modification, probably phosphorylation. Phosphorylation and dephosphorylation reactions cause acidic or basic shifts in pI, respectively and could explain the large numbers of protein isomers in NM from stimulated lymphocytes.

• NHP phosphorylation. HeLa NM proteins are phosphorylated differentially throughout the cell cycle by an endogenous matrix-associated protein kinase (Henry and Hodge, 1983). Many of these phosphoproteins are maximally modified in S phase, with minimal phosphorylation in mitosis and early G1 (Song and Adolph, 1983). In lectin-stimulated lymphocytes high levels of nuclear NHP phosphorylation in the first 20-24h ps are directly related to elevated rates of transcription and RNA processing (Kostka and Schweiger, 1985; Schweiger and Kostka, 1984). Since transcription and RNA processing reactions are reported to occur at the matrix (see Literature Review), it is plausible that NHP

phosphorylation modulates interactions between the nuclear matrix and RNA/P. In addition there could be effects on chromatin-NM interactions during interphase. A third effect of NHP phosphorylation is on protein-protein interactions. For example, phosphorylation of lamins results in appearance of numerous charge isomers during interphase (Gerace and Blobel, 1981; Kaufmann et al, 1983). Reversible lamina depolymerization before mitosis is accompanied by greatly elevated levels of lamin phosphorylation (Gerace et al, 1984). Similarly, lamina reorganization after mitosis is preceded by dephosphorylation of lamins. It is likely that enzymic modification of other matrix NHP during interphase is responsible for reversible localized modulations of nuclear matrix functions. This could account for the large number of putative phosphoproteins in lymphoblast NM.

In summary it appears that more complex polypeptide compositions of NM from con A-stimulated (vs. dormant) bovine lymphocytes arises from (1) ~~de novo~~ synthesis of proteins and (2) post-translational modification. This could be elucidated by further studies involving 2-D PAGE and autoradiography of (eg. ³⁵S-met or ³²P-phosphate) pulse-labeled lymphocyte NM proteins.

Not all lymphocytes in a culture are stimulated by con A, as 10-20% of the population at 36h ps is type 1

cells. The effect of this asynchrony is to decrease the magnitude of changes observed during cell stimulation using averaging techniques such as biochemical extraction. When nuclei are classified individually by microscopy a clearer separation of data is obtained (Hall, 1982). However, proteins unique to -con A matrix are absent from or insignificant in matrix preparations after con A stimulation indicating that the background of type 1 cells present is probably not an important factor in assessing the effect of stimulation. Regardless of this potential background problem it is clear that striking compositional changes occur in nuclear matrices during con A stimulation, coincident with elaboration of nuclear matrix structure, particularly the IM.

(iii) Nuclear Lamins

Lamins A and C are not apparent in gels of -con A matrix, even after silver staining. On the other hand, lamin B is a major component of lymphocyte NM ($\pm$ con A). The absence of lamins A and C from gels of NM from unstimulated lymphocytes was also observed by Nakayasu and Ueda (1981, 1983). Lamin B is also more concentrated than A or C in rat liver NM or lamina (Peters and Comings, 1980). By 18h ps with con A a group of five spots appears near 75C (72C or 76C at 36h ps). The relative positions of the polypeptides suggests that this may be lamin A.

(Gerace and Blobel, 1981; Kaufmann et al, 1983). Similarly a group at 63C-B, at 18h (63-65C at 36h) may be lamin C. Because of abundant intranuclear material in these matrices the lamins represent a minor subset of total NM protein, an observation reported by Peters and Comings (1980), Peters et al (1982), Bouvier et al (1985a) and Bereznay (1984). All in situ lymphocyte nuclear morphotypes stain strongly with antisera against lamin B while lamins A and C are weakly stained (N. Chaly, personal communication). These data are consistent with the results obtained from 2-D gels of NM (this study). Thus the low relative concentrations of A and C likely did not result from their extraction during isolation of lymphocyte NM.

Extreme condensation of lymphocyte peripheral heterochromatin may preclude a requirement for lamins A and C in the nuclear envelope. There is significant rearrangement of condensed chromatin in types 2 and 3 lymphocyte nuclei. The appearance of lamins A and C in NM after con A stimulation could be related to their putative DNA-binding activity (Comings and Wallack, 1978; Hancock and Hughes, 1982) or to increased amounts of PCL in lymphoblast-NM. The lamina becomes more distinct in sections of intact types 2 and 3 cells when observed in the EM, perhaps correlating with the appearance of lamins A and C. If lamins are partially responsible for

organization of the nuclear envelope (Gerace and Blobel, 1981; Lebel and Raymond, 1984) it is plausible that they could also aid in increasing nucleo-cytoplasmic exchange in stimulated cells.

(iv) Other Nuclear Matrix Proteins

The identities of some other nuclear matrix polypeptides have been proposed by previous workers using other cell types. Rat liver hnRNA-associated proteins migrate primarily between 31k and 44k with net basic charge in NE gels. These species account for up to 35% of the total protein mass of rat liver nuclear matrix (Peters and Comings, 1980). Van Eekelen and van Venrooij (1981) showed that very basic 40-44k hnRNP core proteins bind hnRNA to the nuclear matrix. Corresponding lymphocyte protein families included the major series at 34 0-C to 36 0-C, as well as 43.5C-B, 41Hn-C, 39C-B, 36C-B, 35C-B and 33C. The series at 39 to 40 0-Hn were not found in liver but were likely also RNA-associated. Bouvier *et al* (1985a,b) found that extensive RNase digestion of HeLa nuclei released polypeptides similar to lymphocyte 49 0-Hn. An acidic protein at 75A could be associated with poly (A) tails of processed hnRNA (Peters and Comings, 1980; Maundrell *et al*, 1981), binding pre-mRNA to nuclear matrix (Herman *et al*, 1978). It is clear that transcription-associated proteins are found with the

nuclear matrices, particularly in NM from mitogen-activated cells in which transcription and RNA processing are stimulated several-fold. These components may be anchored to an underlying proteinaceous nucleoskeleton as envisaged by Brasch (1982) and Agguter (1985).

Minor groups of 2-4 spots near 61-62A at 36h ps were similar to Q1 and Q2 families found in rat liver matrix. These were presumed to be derived from duplicated genes. No functional significance has been ascribed to these proteins (Peters and Comings, 1980).

In addition to the lamins other protein families were found between 68-80k in bovine lymphocyte NM. These polypeptides, more basic than the lamins, were located at 68Hn-C to 81Hn-C in NM from all 3 of the lymphocyte populations. The presence of 5 to 6 evenly spaced isomers at each MW suggested post-translational modifications. As the isomers had nearly identical pI in each family the polypeptide sequences of different families may be similar. Additional clusters of more basic polypeptides at 68 O-Hn and 73 O-Hn in 18h NE gels were minor components. Similar proteins were found in rat liver nuclear matrix (Peters and Comings, 1980). The exact identity of these proteins is unknown, although they are distinct from the lamins immunologically (Kaufmann and Shaper, 1984; Berezney, 1984) and by peptide mapping (Kaufmann and Shaper, 1984). These proteins are major

intranuclear matrix components of rat liver (Berezney, 1984; Peters and Comings, 1980) and can be released from nuclei using sulphydryl blocking reagents. This indicates potential stabilization of these intranuclear proteins by intermolecular disulphides (Kaufmann and Shaper, 1984). Bouvier *et al* (1985a,b) released apparently similar proteins from HeLa nuclei by extensive RNase digestion and presumed an association with RNA. This may be plausible due to the basic charge of these proteins, although this has not been shown. Alternatively these proteins may mediate DNA binding to nuclear matrix. More of these proteins are present in NM from stimulated lymphocytes, suggesting an association with elevated nuclear metabolism and/or elaboration of NM structure.

Another major protein released from rat liver nuclei by sulphydryl blocking reagents was an acidic species at 38k (Kaufmann and Shaper, 1984). In mouse lymphocyte NM (Setterfield *et al*, 1985) and bovine lymphocyte NM (this study) a very similar polypeptide (38A-S) increases dramatically in relative concentration during con A stimulation. It also undergoes slight basic pI shifts at 18h and 36h ps. No function has been determined for this presumed intranuclear protein to date.

F. Possible Roles for NM in Stimulated Lymphocytes

Previous studies have ascribed numerous functions to the nuclear matrix, including DNA attachment and replication (Berezney et al, 1979; Smith et al, 1984; Berezney, 1984), an association with transcribed genes (Jost and Seldran, 1984; Small et al, 1985) and with RNA metabolism (Jackson and Cook, 1985; Miller et al, 1978a,b; Mariman et al, 1982; Agutter, 1984). In this regard it is not surprising that matrices from unstimulated lymphocytes have simple structure and composition. Conversely NM from lymphocytes 18h or 36h ps are complex in structure and composition as transcriptional rates accelerate and cells prepare for and undergo DNA replication. Analysis of 2-D gels of NM from the latter cells indicates the presence of numerous RNA-associated proteins and intranuclear matrix proteins. Specific DNA-associated NHP have not yet been identified in 2-D gels so their presence or absence is unclear.

In earlier studies comparisons of NM proteins from active and inactive cells showed no significant differences by 1-D PAGE (Hodge et al, 1977; Berezney et al, 1979a; Kuzmina et al, 1984). In contrast, marked changes are observed in the protein profiles of NM during mitogenic stimulation of bovine lymphocytes as these cells proceed from an inactive G0 state to a very active S phase within 24-28h. The nuclear matrix appears to be a dynamic

structure during this period. In lymphocytes, progression through the cell cycle after lectin stimulation is accompanied by more substantial morphological changes than are observed in many other cell types. Small quiescent nuclei are transformed into very large, active, blast nuclei. Coincident with increased complexity and amount of IM *in situ* there is far greater polypeptide complexity in isolated lymphocyte NM. As discussed above, changes in polypeptide composition are likely related to structural and/or functional phenomena in stimulated lymphocytes. Activation of NM-associated processes (eg. DNA replication, transcription and RNA processing and transport) is reflected in observed changes in nuclear matrix structure and composition. Whether these processes cause, or are the result of, structural changes in NM is unknown. Several structural proteins are probably synthesized and incorporated into the nuclear matrix during mitogenesis. Roles for many of the numerous polypeptides revealed in 2-D gels are unknown. One approach to determining structure-function relationships in NM is development and application of immunological probes. Examples of such molecular probes are discussed in Chapter 2 and give further evidence of an intranuclear matrix in lymphocytes which undergoes cell cycle-dependent modulations in structure and function.

CHAPTER TWO

MONOCLONAL ANTIBODIES AGAINST NUCLEAR MATRIX

INTRODUCTION

The nuclear matrix (NM) is a residual nuclear fraction which resists nuclease digestion and extraction by low and high ionic strength and consists of 3 interconnected structures; namely, the pore complex-lamina, residual interchromatinic material (IM) and residual nucleolus (Berezney and Coffey, 1977). The intranuclear matrix is a structurally complex network which resembles interchromatinic material within nuclei in situ (Brasch, 1982; Setterfield *et al.*, 1985). The nuclear matrix is a fraction of complex polypeptide composition (Peters and Comings, 1980; Peters *et al.*, 1982; Chapter 1). Only a small number of proteins identified in 2-D gels have been isolated and characterized to date.

Many nuclear metabolic processes have been localized within the nuclear matrix (Literature Review), usually through gross biochemical analyses of NM preparations. Only rarely have correlations between the matrix and nuclei in situ been shown (eg. Smith *et al.*, 1984). The nuclear matrix remains a controversial structure whose reality in situ has been challenged (eg. Kaufmann and Shaper, 1984; Bouvier *et al.*, 1985a). Unequivocal demonstration that the NM is real or artifact has yet to be made, such that several aspects of nuclear matrix structure and function must be clarified. The actual

proteins of an *in situ* NM must be identified, and their persistence in isolated matrices, or their extraction from nuclei must be shown. Structural vs functional proteins must be identified, although proteins such as topoisomerase 2 appear to be both (eg. Berrios *et al*, 1985). The cell cycle distributions and amounts of individual proteins must also be determined, in an attempt to correlate particular proteins with possible roles. One approach to describe particular components of the nuclear matrix is to create a library of immunological probes against NM proteins. When these studies were initiated in 1982 only the lamins (Gerace and Blobel, 1981) and hnRNP core proteins had been isolated and well characterized. Since then other studies using antibodies against NM proteins have been reported (eg. Chaly *et al*, 1984; Woodcock *et al*, 1984; Berrios *et al*, 1985).

Monoclonal antibodies are useful in studying complex protein mixtures because discrete antigens can be identified without purifying individual proteins from a mixture for injection into animals. In this study monoclonal antibodies were raised against lymphocyte DNase- digested nuclear matrix and were partially characterized. Antigens were localized by immunofluorescence. Distributions of antigens were compared in cells, nuclei, extracted nuclei and nuclear matrices. Changes in amounts and distributions of

antigens were found throughout interphase and mitosis in con A-stimulated lymphocytes. In addition, the evolutionary conservation of the antigens was partially summarized. The results of this study suggest that the nuclear matrix is a real structure, albeit one which is variably modified during its isolation. It is suggested that caution is necessary when ascribing specific roles to nuclear matrix-associated proteins or nucleic acids.

MATERIALS AND METHODS

A. Cell Culture

Mouse splenocytes were isolated and cultured as described previously (Setterfield et al, 1983). Bovine retropharyngeal lymph node cells were isolated and cultured as described in Chapter 1. Mouse 3T3 and rat kangaroo PtK cells were cultured at 37 C with 5% CO₂ in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal calf serum (FCS), 100U/ml penicillin and 100ug/ml streptomycin. Mouse myeloma Sp2/0 cells were grown in the same medium with 10% CO₂.

B. Isolation of Nuclear Matrix

Free nuclear matrices were prepared from isolated nuclei by the method described in Chapter 1. Nuclear matrices *in situ* were prepared by passing unhomogenized cells through the same isolation procedure. Cells were extracted for 20 min at room temperature in 1% Triton X-100 (v/v) in STM buffer. They were subsequently digested in DNase 1 (10ug/ml) for 20 min at room temperature. Extractions in 1M HSB and 2M HSB were 15 min each on ice. A final nuclease digestion in DNase 1 ($\pm$ RNase A: 50ug/ml each) was 20 min at room temperature. Cultured 3T3 or PtK cells were similarly extracted by passing coverslips on which they were grown through the

same extinction sequence.

C. Production of Monoclonal Antibodies

Nuclear matrices isolated from resting bovine lymphocytes or mouse splenocytes were used as immunogens in different experiments. Each of 4 female Balb c/BYJ mice (from Jackson) received three intraperitoneal injections of 400ug immunogen at ten day intervals, followed by a 100ug intravenous injection ten days later. Three or four days later splenocytes isolated from the immunized mice were fused with mouse myeloma Sp2/0 cells using polyethylene glycol (PEG), according to the method of Kennett (1980a). The fused cells were grown in six 96-well plastic culture plates in HY medium (Appendix 5) at 37°C with 10% carbon dioxide. Hybridomas were selected by use of the HAT protocol (Appendix 5). Ten to 14 days after fusion supernatants were removed from culture plate wells which sustained growth of hybrid cells and assayed for antibody production (see below). Hybridomas in positive wells were cloned once by limiting dilution (McKearn, 1980) and once in semi-solid agar or twice in semi-solid agar (Kennett, 1980b). Cloned cells were tested after each cloning step for antibody production. In addition, cells were frozen at -80°C at each stage.

Antibodies were amplified through production of ascites fluid. Female Balb c/BYJ mice were given an

intraperitoneal injection (1 ml/mouse) of 2,6,10,14-tetramethylpentadecane (pristane). After 7 days pristane-primed animals received intraperitoneal injections of 1 million hybridoma cells in DMEM. Ascites fluid was collected between 7 and 10 days after injection, centrifuged at 1000 x g for 10-20 min to pellet erythrocytes, other cells and insoluble material, and stored at 4°C with 0.2% sodium azide to inhibit bacterial growth.

D. Screening of Monoclonal Antibodies

Typically six 96-well culture plates were used to grow the fusion products from each experiment. In order to quickly select the few antibody-producing cell lines a rapid assay procedure (D.L. Brown, personal communication) was used, allowing all interesting hybridoma lines to be selected in one day. Mouse 3T3 cells grown on 35 x 50mm glass cover slips were fixed in 95% ethanol for 15 min at 4°C, followed by air drying for 20 min. Each dried coverslip was oriented over a cardboard template having an evenly spaced 6 x 4 matrix of dots and one corner of the coverslip was marked. A 2ul drop of culture supernatant from each well containing growing hybridoma cells was placed over a dot. After a 30 min incubation at room temperature the coverslip was rinsed 3 x 4 min in phosphate-buffered saline (PBS), pH 7.0. The coverslip

was then flooded with 400ul of fluorescein isothiocyanate (FITC)- conjugated goat anti-mouse Ig (Cappel Laboratories) and incubated for 30 min at room temperature. The coverslip was rinsed 3 x 4 min in PBS and mounted on a 50 x 75mm slide in 30ul of 0.1% (w/v) p-phenylenediamine in 50% (w/v) glycerol in PBS, pH 7.0. Using the cardboard template, the locations of supernatant drops were circled using a marking pen. Areas of the coverslip between supernatants acted as controls showing non-specific background fluorescence. Areas within supernatant spots were observed to determine specific fluorescence and staining pattern.

E. Immunofluorescence Microscopy

Cultured 3T3 or PtK cells used for microscopy were grown on sterile coverslips for immunostaining. Lymphocytes, isolated nuclei and nuclear fractions were attached to coverslips for microscopy. Clean 22 x 22mm glass coverslips were coated with 0.1% (w/v) poly-L-lysine (in distilled water) for 2 min and rinsed by dipping several times in distilled water. After air drying coverslips were flooded with 100ul of sample and incubated for 10 min at room temperature. All subsequent steps were performed at room temperature in PBS, pH 7.0, with supplements as noted. The coverslips were washed in PBS (2 x 30 sec), fixed for 5 min in 3% (w/v)

paraformaldehyde, washed (2 x 30 sec) and reduced in sodium borohydride (1 mg/ml; 3 x 4 min) to block aldehyde autofluorescence. Cells or nuclei were treated with 0.2% (v/v) Triton X-100 for 20 min, followed by PBS washes (3 x 4 min). Coverslips were then flooded with 75ul of monoclonal antibody at the following dilutions: P1, 1:250; I1, 1:100; P11 and P12, 1:500. Control samples were flooded with 75ul of PBS. Samples were incubated at room temperature in a humid chamber for 45 min. After more PBS washes (3 x 4 min), 75ul of FITC-conjugated goat anti-mouse Ig antibody (1:75 in PBS; Cappel Laboratories) was added. After a 45 min incubation at room temperature in a humid chamber the coverslips were washed (3 x 4 min) in PBS. Most samples were counterstained for DNA by a 2 min incubation in 1.5ug/ml Hoechst 33258 (CalBiochem). The coverslip was mounted on a slide in 6ul of mounting medium (see above). Control samples were routinely examined to ensure that fluorescein staining patterns were genuine.

Samples were observed and photographed in a Zeiss Universal photomicroscope fitted for epifluorescent illumination. FITC fluorescence was detected under filters while Hoechst fluorescence in the same sample was observed with filters. Preparations were photographed on Ilford XP4-400 b/w film and printed on Ilfospeed 45 paper.

F. Monoclonal Antibody Typing

In order to determine the antibody type produced by a particular hybridoma, 3T3 cells incubated with each monoclonal antibody were stained with secondary FITC-conjugated goat antibody specific for either mouse IgG or IgM molecules (Flow Labs). These samples were processed for immunofluorescence microscopy as described above.

G. Immunoblotting

Lymphocyte nuclear matrix proteins were separated in 1-D 10% SDS-PAGE gels as described in Chapter 1. The polypeptides were transferred electrophoretically to nitrocellulose (Bio-Rad; pore size 0.45um) by the method of Burnette (1981) with modifications. The electrode buffer contained 192mM glycine (Bio-Rad) and 25mM Trizma base (Sigma). For direct visualization of transferred polypeptides a strip of nitrocellulose was stained 30 sec in 0.1% (w/v) amido black in 45% (v/v) methanol, 10% (v/v) acetic acid, destained for up to 5 min in methanol-acetic acid and washed in distilled water.

After transfer unstained nitrocellulose was immediately incubated in PBS, pH 7.0, containing 1.5% gelatin (Sigma) for 2-3h to block background protein binding. The sheet was washed in PBS containing 0.1% (w/v) gelatin pH 7.0 for 60 min at room temperature. All

subsequent steps were performed in this buffer at room temperature, unless otherwise noted. Strips of the nitrocellulose (5-10mm wide) were incubated with diluted ascites fluid at the following dilutions: P1, 1:1000; PI1 and PI2, 1:200. Following incubation in a humid chamber (4°C for 16h) the strips were washed sequentially in fresh buffer containing 0.05% (v/v) Nonidet P-40 (2 x 10 min) and lacking NP-40 (2 x 10 min). The strips were then incubated in horseradish peroxidase-conjugated goat anti-mouse IgM (Cappel Laboratories; diluted 1:400) for 60-90 min. The strip was washed as above and developed in 0.03% (v/v) hydrogen peroxide, 0.1mg/ml diaminobenzidine (DAB; Polysciences) in PBS.

RESULTS

A. MONOCLONAL ANTIBODIES AGAINST NUCLEAR ANTIGENS

1. Characterization of Monoclonal Antibodies

From three fusion experiments some 1500-1600 HAT-selected hybridoma lines were screened. Of these, 21 lines secreted monoclonal antibodies recognizing nuclear antigens. These lines were originally detected by immunofluorescence using ethanol-fixed mouse 3T3 cells. Mouse lymphocytes were not used for this purpose since surface Ig on B cells reacted with the secondary antibody

and thymus (T) cells were too small to give useful staining patterns. Many other hybridoma lines produced antibodies against cytoplasmic elements which often appeared filamentous. These were not studied further.

Four monoclonal antibodies which gave strong distinctive staining of nuclei were selected for detailed study. All four antibodies reacted with FITC-conjugated goat anti-mouse IgM but not anti-IgG. These antibodies showed unique staining patterns in intact interphase nuclei of 3T3 cells. These basic patterns have been described in detail (Chaly et al, 1984). Two methods of fixation of 3T3 cells were used. Ethanol fixation was used during initial screening experiments but variable staining patterns were observed. Less staining variability and/or less antigen disruption were observed after formaldehyde fixation, presumably because this agent acts by crosslinking molecules and not by precipitating or denaturing antigens (D.L. Brown et al, 1983; personal communication). The staining patterns observed in intact 3T3 cells are summarized below.

Antibody P1. Antibody P1, produced against bovine lymphocyte nuclear matrix, stained the nuclear periphery exclusively with a uniform brightness (figs. 26a and 26e), regardless of fixation method used. Focussing on the nuclear surface showed a fibrillar pattern of P1 fluorescence (fig. 26i).

Antibody I1. A second antibody against bovine lymphocyte nuclear matrix gave bright punctate, non-nucleolar staining of the nuclear interior with no staining of the nuclear periphery (figs. 26b, 26f). Antibody I1 stained small interior speckles of uniform size and intensity, although these were smaller in formaldehyde-fixed cells.

Antibody PI1. Antibody PI1 was produced against mouse lymphocyte nuclear matrix and in the majority of nuclei stained uniformly sized peripheral granules, in addition to intranuclear staining. The latter consisted of non-heterochromatic, non-nucleolar granules of variable size and intensity. These were arranged in irregular clusters throughout the nuclear interior (figs. 26c and 26g). Similar results were obtained regardless of fixation method used. Antibody PI1 recognized large granules in an apparent fibrogranular network at the nuclear periphery (fig. 26j).

Antibody PI2. Antibody PI2 was also produced against mouse lymphocyte nuclear matrix and gave similar staining of 3T3 cells as PI1 after ethanol fixation (fig. 26d). However, the peripheral nuclear stain consisted of finer granules, arranged unevenly, and the small, uniformly sized intranuclear granules were distributed more evenly than those stained by PI1. In formaldehyde-fixed 3T3

cells PI2 gave only bright peripheral staining in some nuclei, while in other nuclei this was accompanied by small intranuclear non-nucleolar granules. In general there were fewer intranuclear granules stained by PI2 than by PI1 (fig. 26h). In addition, PI2 recognized smaller granules at the nuclear surface than PI1, and these were more uniformly arranged (fig. 26k).

Results from immunoblotting of 1-D SDS-PAGE of lymphocyte nuclear matrix polypeptides are shown in Figure 27. Antibody P1 recognized a doublet or triplet at 27-30k. PI1 stained a protein with MW 60k and PI2 stained bands at 130k, 70k and 35k. These results were reproducible and have been confirmed using total nuclear protein and by different laboratories (not shown). Antibody I1 did not give conclusive or consistent results by immunoblotting, regardless of the conditions or antigen used. It is not known if this antigen was extremely labile but incubations at 4°C in the presence of protease inhibitors were negative also. It is possible that the antigenic determinant recognized by I1 may have been sensitive to SDS denaturation. However, extensive washings of the nitrocellulose strips or use of an ELISA-derived detection method did not alter the results.

Figure 26. Immunofluorescence labelling of interphase 3T3 cells by antibody P1 (a,e,i), I1 (b,f), PI1 (c,g,j) and PI2 (d,h,k).

Figure 26a-d. Ethanol-fixed 3T3 cells labelled with undiluted hybridoma supernatants (x 1800).

Figure 26e-h. Formaldehyde-fixed, Triton-permeabilized 3T3 cells stained with diluted ascites fluids (x 1200).

Figure 26i-k. Focus at nuclear surface of 3T3 cells stained as in Fig. 26 e,g and h, respectively (x 1200).

Figure 27. Immunoblotting of monoclonal antibodies against bovine lymphocyte nuclear matrix polypeptides. Lane A: Total protein transferred to nitrocellulose stained with amido black; Lanes B-D: Antigens detected by antibodies P1 (lane B), PI1 (lane C) and PI2 (lane D). Molecular weights of protein standards (left) and of antigens (right), in kd.

2. Conservation of Nuclear Antigens

— Table 5 lists the staining patterns of several diverse cell types using the monoclonal antibodies prepared against lymphocyte nuclear matrix. Antibodies P1 and I1 stained every cell type tested, i.e. mammalian, avian, insect, plant, fungus and unicellular flagellate cells, in the same manner as in 3T3 cells. The antigens recognized by these antibodies appear to have been remarkably conserved through evolution.

Antibodies PI1 and PI2 gave somewhat variable results in the cell types listed in Table 5. Mammalian cells and the Drosophila cell line gave "typical" peripheral and internal staining of nuclei. Nuclei of more evolutionarily distant species were stained with neither PI1 nor PI2. Most mammalian cells and Drosophila KcA311 had peripheral and internal nuclear staining, but exceptions were noted. Mouse lymphocytes (clone A) had only peripheral nuclear staining with PI2 whereas PtK nuclei were only internally stained. E. acrea haemocytes were devoid of PI1 labelling in the nuclear interior. While a diverse range of cell types are stained the results with PI1 and PI2 suggest that the recognized antigens are less highly conserved than those of P1 and I1 and may show somewhat different distributions in nuclei of related species or different tissues of the same species.

TABLE 5 - Localization of Monoclonal Antibodies at Interphase

Cell Type	Antibody *			
	P1	I1	PI1	PI2
	(bovine)	(bovine)	(mouse)	(mouse)
Mouse 3T3	P	I	P+I	P+I
Mouse oocyte	P	ND	ND	ND
Mouse sperm	P	ND	ND	ND
Mouse lymphocyte	P	I	P+I	P
Bovine lymphocyte	P	I	P+I	P+I
Human Lymphocyte	P	I	P+I	P+I
HeLa	P	I	P+I	P+I
Chinese hamster				
ovary (CHO)	P	I	P+I	P+I
PtK2	P	I	P+I	I
Chick-reactivated				
erythrocytes	P	ND	ND	ND
Sea urchin oocyte	P	ND	ND	ND
Sea urchin sperm	P	ND	ND	ND
<u>Drosophila</u> KcA311	P	I	P+I	P+I
<u>Estigmene acrea</u>				
(insect) haemocytes	P	I	P	P+I

TIV cytoplasmic

assembly centers **	+	+	-	-
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Vicia hainiana

(bean)	P	I	-	-
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<u>Entomophthora</u> (fungus)	P	I	-	-
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<u>Polytomella agilis</u>	+	+	-	cytoplasm
(unicellular flagellate)				

* - Source of the original nuclear matrix immunogen for each antibody is given in brackets.

P indicates peripheral nuclear staining; I indicates intranuclear stain; ND=not determined; "-" = negative; "+" = positive.

** TIV = Tipula Iridescent Virus (in T. acraea cells);

Bladon et al, manuscript in preparation. Results in HeLa, CHO, KcA311 and Entomophthora - N. Chaly, personal communication. Staining of meiotic tissues - Schatten et al (1985). Staining of chick erythrocytes - C.L.F. Woodcock, personal communication. Staining of Vicia - M.P. Sabour, personal communication. Staining of Polytomella - W. Aitchison, personal communication.

3. Immunostaining of Lymphocytes

(a). Antibody P1

When unstimulated mouse splenocytes were stained with P1, the peripheral nuclear staining closely followed the clefting of the nuclear membrane (fig. 28a-c).

Additionally, a minor staining component, visible just inside the nuclear membrane, resembled fine strands which appeared to be continuous with, but perpendicular to, the nuclear periphery. This was not observed in stimulated lymphocyte nuclei. No intranuclear or cytoplasmic staining was observed. Nuclei in con A-stimulated mouse lymphocytes also showed peripheral nuclear staining (fig. 28c) except that it was far brighter than in -con A cells. Photographic print exposures which showed details in type 3 nuclear staining resulted in negligible intensity of adjacent type 1 nuclei (see also Table 6). Bovine lymphocytes stained with P1 showed fluorescence similar to mouse splenocytes, with a fairly uniform granular staining of the nuclear periphery (fig. 29). As in mouse cells nuclear clefting was clearly visible in unstimulated cells. Lymphoblast nuclei were rarely clefted.

During mitosis in mouse splenocytes (48h ps) the distribution of P1 antigen was markedly altered. In early prophase, as chromosomes condensed near the nuclear membrane, stain appeared around the chromosomes but

remained continuous with the nuclear envelope (fig. 30a,b). Later in prophase the chromatid arms were resolved (fig. 30c,d) and P1 antigen appeared at the edges of the chromosomes, coating the individual arms. The intensity of chromosomal stain was much greater than interphase nuclei, even type 3, which often were not visible in prints showing prophase nuclei. The perichromosomal distribution of P1 antigen remained the same during prometaphase and metaphase although the intensity of staining decreased slightly. No cytoplasmic stain was visible. To this point in mitosis P1 stained material which rimmed the chromosome mass. During anaphase individual sets of daughter chromosomes were surrounded by P1 antigen. Individual chromosome arms which extended from the chromosomal masses showed a thin rim of perichromosomal stain (fig. 30h). In telophase (fig. 30j), prior to decondensation, the chromosomes formed condensed "nuclei" in which P1 staining was confined to the periphery, with an uneven distribution following the outline of the chromatin (compare FITC and Hoechst; figs. 30i and 30j). In early G1, as interphase nuclei reform, the nuclear periphery remained brightly stained with a slight amount of internal staining between peripheral condensed chromatin masses. Although brighter, in general the pattern was similar to that in freshly isolated unstimulated cells. The mitotic distribution of

P1 has been confirmed in several cell types, including 3T3 and PtK (Chaly *et al.*, 1984).

(b) Antibody I1

Formaldehyde-fixed lymphocytes stained with antibody I1 showed distributions similar to those in 3T3 or PtK cells. Intranuclear speckles of various sizes (non-nucleolar) were stained. As the fluorescein and Hoechst fluorescence patterns were different, it appeared that the interior speckles were also non-heterochromatin.

Unstimulated populations of mouse or bovine (fig. 29f)

lymphocytes were heterogeneous with regard to stain intensity. Some nuclei did not stain visibly while others were stained lightly. This may have been due to cytoplasmic factors since Triton nuclei stained relatively homogeneously (see Part B). Types 2 and 3 nuclei stained consistently and progressively brighter than type 1 nuclei (fig. 29r, also Table 6). Some cytoplasmic stain was visible at times, although this was slight when compared to nuclear staining.

In early prophase in mouse lymphocytes, as chromosomes condensed near the nuclear periphery, granular I1 stain followed the Hoechst DNA stain (fig. 30-1). This continued through prometaphase where the chromosome mass stained uniformly with I1. Where individual chromatid

arms were resolved they showed granular staining throughout (fig. 30n). The intensity of mitotic stain was approximately the same as in interphase. Chromosomal staining by I1 remained the same during metaphase (fig. 30p) and anaphase (fig. 30r). As the nucleus reformed in telophase it was difficult to resolve individual chromosomes but, as in prophase, I1 staining followed the Hoechst pattern (compare figs. 30s and t), suggesting an association of I1 antigen with chromatin. These distributions of I1 antigen during mitosis have been confirmed in 3T3 and PtK cells (Chaly et al, 1984).

(c) Antibody PI1

When nuclei in unstimulated mouse or bovine cells were stained with antibody PI1, intranuclear granules were the primary component labelled; these were most often faint, although a few bright granules were also seen (figs. 28i and 29i). These were apparently non-heterochromatic and non-nucleolar. Unlike 3T3 or PtK cells peripheral nuclear granules were infrequent in con A lymphocytes. Morphotypes 2 and 3 nuclei present in cultures stimulated for 48h by con A had different staining characteristics (see Table 6). These nuclei stained much brighter than type 1 and had a uniform rim of large bright peripheral granules (fig. 29 u). In addition there were more numerous smaller intranuclear granules.

During early prophase two very brightly stained granular patches appeared at sites on the nuclear periphery which appeared to be invaginated. No intranuclear fluorescence was detected (fig. 31b). This rearrangement in antigen distribution has been observed in 3T3 cells also (Chaly et al, 1984). In prometaphase, after breakdown of the nuclear envelope, faint fluorescence was sometimes observed in the area between the oriented chromosomes (fig. 31f). In lymphocytes this was often not clearly resolved. On the other hand, in PtK cells this area remained strongly stained in prometaphase (fig. 31h). PI1 staining of mouse lymphocytes in metaphase was restricted to very weak granular staining of the cytoplasm. The chromosomes aligned at the metaphase plate were unstained (fig. 31j). The brightness and resolution of the metaphase staining were more clearly seen using PtK cells (fig. 31 l). The distribution of PI1 antigen was unchanged in anaphase (figs. 31n and p). As shown in fig. 31r diffuse fluorescence was found near the spindle poles in telophase, although this was not as clearly resolved in lymphocytes as in 3T3 (Chaly et al, 1984) or PtK (fig. 31t) cells. In early G1, as nuclei reformed, PI1 reappeared at the nuclear surface with some intranuclear granules also stained. In general mitotic lymphocytes stained diffusely and had less intense fluorescence when compared to other cell types (see above). Adjacent interphase cells had

typical staining intensities.

(d) Antibody PI2

Mouse lymphocytes stained with antibody PI2 had a unique pattern of fluorescence (fig. 28 l). All 3 nuclear morphotypes were brightly stained, exclusively at the nuclear periphery. Nuclear invaginations were clearly visible. This staining pattern was similar to P1. Bovine lymphocytes stained with PI2 had a more "typical" pattern of immunofluorescence (fig. 29 l). Type 1 nuclei had a fine granular nuclear peripheral stain with a few internal granules present. These nuclei were heterogeneous with regard to staining intensity; some nuclei were strongly stained while others had weak immunofluorescence. Types 2 and 3 nuclei were stained brighter than type 1 (fig. 29x; see also Table 6). Nuclear peripheral staining was much stronger and there were more intranuclear granules. The latter were not as numerous as in bovine lymphocytes stained with PI1 or 3T3 cells stained with PI2.

In mitotic mouse lymphocytes the nuclear periphery remained stained in early prophase although the intensity decreased (fig. 32b). Intranuclear granules were not easily resolved in bovine lymphocytes. In 3T3 or PtK cells two brightly stained granular patches were found at nuclear invaginations later in prophase (fig. 32d) but such patches were not detected in lymphocytes. In

prometaphase faint stain appeared between chromosome ends and in the cytoplasm (fig. 32f). In PtK cells the stain between chromosome ends was much brighter than in interphase (fig. 32h). In metaphase and anaphase cells granules of approximately interphase size were stained in the cytoplasm. In some cases there was a slightly brighter patch near chromosomes in the same plane as the spindle poles (fig. 32j). The chromosomes remained unstained. In telophase considerable cytoplasmic stain remained. In addition there was often slightly brighter staining around the edges of chromosome masses (fig. 32r). As shown in fig. 32t perichromosomal staining by PI2 was relatively bright in PtK cells. By early G1 in lymphocytes there was fairly intense staining of the nuclear envelope, although not as bright as in interphase nuclei. Again PtK cells were far brighter at this stage than mouse lymphocytes.

Figure 28. Distributions of nuclear antigens in mouse splenocytes 48h ps with con A. Immunofluorescent labelling of (c) P1 antigen, (f) I1 antigen, (i) PI1 antigen and (l) PI2 antigen with monoclonal antibodies. Corresponding cells are shown by phase contrast microscopy (a,d,g,j, respectively) and DNA is labelled with Hoechst (b,e,h,k, respectively). Types 2 and 3 cells are stained brighter than small type 1 nuclei with each of the antibodies. A type 1 nucleus stained with I1 (fig. 3f; arrow) shows a nonheterochromatin staining pattern (x 1650).

Figure 29. Distributions of nuclear antigens in unstimulated bovine lymphocytes (a-l) and in lymphocytes 36h ps with con A (m-x). Phase contrast micrographs (column a-v), fluorescent labelling of DNA with Hoechst (column b-w) and immunofluorescent labelling of antigens with monoclonal antibodies (column c-x). (a-c) P1 staining of unstimulated lymphocytes. Antigen closely follows nuclear clefts and is located at the nuclear periphery. (d-f) Unstimulated lymphocytes stained with I1. Note heterogeneous staining of population. Some nuclei are weakly stained while others are relatively bright. (g-i) Unstimulated cells stained with PI1 show primarily intranuclear staining of granules. (j-l) PI2 stains intranuclear and peripheral nuclear granules in unstimulated lymphocytes.

(m-o) P1 stains lymphoblast nuclei brighter than small lymphocyte nuclei; located at the nuclear periphery exclusively. (p-r) I1 stains intranuclear granules within all nuclear morphotypes; stain is not located over nucleoli or heterochromatin. (s-u) PI1 stains peripheral and internal nuclear granules in stimulated lymphocytes. Larger stimulated nuclei are stained brighter than type 1 nuclei. (v-x) PI2 stains peripheral and internal nuclear granules in all 3 nuclear morphotypes. Larger nuclei are often stained brighter (x1600).

Figure 30. Mitotic distributions of P1 and I1 antigens in mouse splenocytes 48h ps with con A. Cells labelled with P1 (column b-j), I1 (column l-t) and counterstained for DNA using Hoechst (columns a-i and k-s, respectively).

Note peripheral nuclear invaginations stained by P1 (a,b) in prophase as chromosomes condense. Individual chromosomes are enveloped by P1 in prometaphase (c,d). This arrangement continues through metaphase (e,f) and anaphase (g,h). Note end-on view of chromosome in anaphase (at left) showing perichromosomal location of antigen. Telophase distribution of P1 (i,j) as daughter nuclei form; P1 stains the convoluted periphery of the condensed chromatin (x 1750).

Antibody I1 stains condensing chromosomes in prophase (k,l) and prometaphase (m,n). There is slight cytoplasmic background. Individual chromatids can be resolved in metaphase (o,p; lower right). This granular antigen distribution continues through anaphase (q,r). During telophase (s,t) most I1 staining is restricted to granules in daughter nuclei but individual chromosomes are not resolved (x 1750).

Figure 31. Mitotic distributions of PI1 in mouse splenocytes 48h ps with con A (columns a-q, b-r) and in PtK cells (columns c-s, d-t). DNA is labelled with Hoechst in columns a-q and c-s.

Few peripheral nuclear granules are stained in lymphocytes in prophase (a, b), although a bright cluster is visible. Only cytoplasmic labelling by PI1 is observed in prometaphase (e, f), metaphase (i, j) and anaphase (m, n). In telophase (q, r) peripheral areas of daughter nuclei are weakly labelled. Dark spots over cells in j-r are optical artifacts. (x 1750).

Mitotic PtK cells were stained more brightly than lymphocytes with PI1. Bright granular nuclear patches are stained in prophase (c, d). Prometaphase cell (g, h) has 2 brightly stained nuclear invaginations and few peripheral granules. Chromosomes are unstained in metaphase (k, l) and anaphase (o, p); cytoplasmic granules are brightly stained in anaphase. In telophase (s, t) there is faint cytoplasmic label and bright staining of granules at peripheries of forming daughter nuclei. (x 1750).

Figure 32. Mitotic distributions of PI2 in mouse splenocytes 48h ps with con A (columns a-q, b-r) and in PtK cells (columns c-s, d-t). DNA was labelled with Hoechst (columns a-q, c-s, respectively).

In mouse lymphocytes in prophase (a, b) PI2 is located at the nuclear periphery, external to condensing chromosomes. By prometaphase (e, f) PI2 antigen has become largely cytoplasmic, with faint labelling of the area between chromosome ends. There is slight staining of PI2 antigen at spindle poles in metaphase (i, j). In anaphase a small amount of PI2 is stained at edges of chromosome masses (m, n), and peripheral nuclear granules are more clearly resolved in telophase (q, r).

In PtK cells the rearrangement of PI2 is more marked in prophase (c, d) and prometaphase (g, h). The unstained chromosome mass is clearly silhouetted against the stained cytoplasm in metaphase (k, l) and anaphase (o, p). By telophase (s, t) the amount of cytoplasmic stain is reduced and daughter nuclei are strongly labelled (x 1750).

TABLE 6 - Immunostaining of Bovine Lymphocytes
with Monoclonal Antibodies

Cell Type	Antibody			
	P1	I1	PI1	PI2
1	P+ I+ Smooth	I+ Fine Granules	P+ I++ Coarse Granules	P++ I+ Fine Granules
3	P+++ Smooth	I+++ Fine Granules	P++ I+++ Coarse Granules	P+++ I++ Fine Granules

Nuclear morphotypes assessed by phase contrast microscopy and costaining with Hoechst 33258. P=peripheral; I=internal; no. of '+' indicates relative staining intensity.

B. STAINING OF NUCLEAR MATRIX (AND INTERMEDIATE FRACTIONS) WITH MONOCLONAL ANTIBODIES

1. Isolated Lymphocyte Nuclear Matrix

(a) Antibody P1

The distribution of antibody P1 in isolated mouse Triton nuclei ($\pm$ con A) was very different from the pattern within nuclei *in situ*. Staining was confined to non-heterochromatin and non-nucleolar regions of nuclei and formed a reticulate meshwork (fig. 33a-c). This anomalous staining pattern was often observed in samples of Triton nuclei from mouse lymphocytes. As shown in figure 8d-f Triton nuclei from unstimulated bovine lymphocytes also had some internal P1 stain, although this was limited to granular patches between peripheral heterochromatin clumps and occurred near the nuclear envelope. Triton nuclei from populations of cells stimulated for 24-48h with con A were stained only peripherally with P1, with no obvious intranuclear fluorescence detected within any nuclear morphotype (fig. 33g-i; see also Table 7). The following results were obtained using bovine lymphocyte nuclear fractions, unless otherwise noted. In DNase nuclei the distribution of P1 antigen did not appear to change significantly (fig. 33j-l). On the other hand, subsequent extraction of DNase nuclei with low M_{wt} buffer (LMB) led to massive

rearrangement of P1 antigen, presumably as DNA was extracted from nuclei (fig. 33m-o; compare Hoechst fluorescence with fig. 33k). The intensity of staining was lower in LMB nuclei, with most of the antigen located in fine speckles in the nuclear interior but not over nucleoli. Further extraction of LMB nuclei with M NaCl (M HSB) led to nuclear shrinkage and aggregation. Antibody P1 stained irregularly-sized granular patches, in addition to some apparently fibrillar structures, both within the interior of M HSB nuclei. The pattern resembled a fine intranuclear meshwork (fig. 33p-r) which was also found in 2M HSB nuclei (fig. 33s-u) and nuclear matrix (fig. 33v-x).

(b) Antibody I1

The antigen recognized by antibody I1 was also rearranged considerably during isolation of nuclear matrix. Bovine Triton nuclei ($\pm$ con A) contained fewer granules per nucleus and which seemed larger than *in situ* (fig. 34a-f), and showed variable brightness. Digestion of nuclei with DNase 1 caused the intranuclear fluorescence with I1 to become less granular but remain bright (fig. 34g-i). The intensity of I1 staining was, however, greatly reduced in LMB nuclei (as was Hoechst; fig. 34j-l). An apparently random internal and peripheral granular stain remained, with some nucleoli strongly

stained. This pattern of I1 staining was also found in M HSB and 2M HSB nuclei (fig. 34m-r). There was also slight staining of DNA by Hoechst in these residual nuclei but this was abolished by DNase 1. In some DNase matrices residual nucleoli stained, while most matrices were unstained. Digestion of 2M HSB nuclei with RNase A and DNase 1 abolished this residual staining (Chaly et al, 1985).

(c) Antibodies PI1 and PI2

Unlike the antigens recognized by P1 and I1, those recognized by PI1 and PI2 were virtually unaffected by the procedures used to isolate nuclear matrix (Table 7). In general the matrices showed no detectable Hoechst staining and were much smaller in diameter than Triton nuclei, the latter characteristic limiting microscopic resolution of detail. Type 1 matrices stained with PI1 contained a few, primarily internal, granules while type 3 matrices had a coarse granular peripheral stain and intranuclear speckles (fig. 35g-i). These results were basically similar to those with nuclei *in situ*. When samples of Triton nuclei were stained with PI2 the type 3 nuclei were generally brighter than type 1, although the number of intranuclear granules was reduced in each class of nuclei (fig. 35j-o). Nuclear matrices stained with PI2 had a granular peripheral stain which was not as fine as that observed in

situ (fig. 35p-r). Intranuclear granules were only rarely observed in matrices stained with PI2.

2. In situ Nuclear Matrix

It was shown in Chapter 1 that isolation of nuclear matrix from nuclei in suspension caused significant rearrangement of the interchromatinic components, which were presumed to be major constituents of the matrix. When unbroken lymphocytes were extracted using the same protocol, residual nuclear structures closely resembled *situ* nuclei, except that only chromatin appeared to have been extracted, leaving an intact interchromatinic region and nuclear envelope (see Chapter 1). For this reason intact bovine lymphocytes (48h ps) were subjected to a modified matrix isolation procedure in order to determine whether the localizations of antigens detected by the monoclonal antibodies had been altered by isolation of nuclear matrices in solution. When cells were extracted using LMB and HSB, much of the DNA was removed, as shown by decreased Hoechst stain. No DNA was detected in 2M HSB cells or in *situ* matrices. In addition, M HSB caused *situ* nuclei to shrink slightly (see also Chapter 1 for EM), but this effect was not as pronounced as for isolated nuclei. Phase contrast microscopy showed that isolated nuclei in cells were more open in appearance than corresponding structures in solution.

Figure 33. Immunofluorescent labelling of isolated nuclear fractions from bovine lymphocytes by antibody P1. Phase contrast micrographs (column a-v), Hoechst staining of DNA (column b-w) and P1 immunofluorescence (column c-x); all micrographs x 2000, except d-f (x 3200).

a-c. Mouse splenocyte nuclei 48h ps with con A. Note intranuclear localization of stain.

d-f. Staining of Triton nuclei from unstimulated bovine lymphocytes sometimes had a slight internal component.

g-i. Triton nuclei from bovine lymphocytes 36h ps with con A stained similar to nuclei *in situ*.

All samples below were from 36h ps bovine lymphocytes. j-l. DNase nuclei are stained peripherally.

m-o. P1 antigen becomes internal in LMB nuclei. Note reduced Hoechst fluorescence after LMB extraction.

p-r. M HSB nuclei are shrunken and P1 antigen is stained in irregular internal clumps. Note extraction of most DNA after HSB.

s-u. P1 stains irregular clusters inside shrunken 2M HSB nuclei (arrows).

v-x. No DNA remains in DNase-treated matrices. P1 stain resembles 2M HSB nuclei.

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Figure 34. Immunofluorescent labelling of isolated nuclear fractions from bovine lymphocytes by antibody I1. Phase contrast micrographs (column a-p), Hoechst staining of DNA (column b-q) and I1 immunofluorescence (column c-r); all micrographs x 2000, except where noted.

a-c. Triton nuclei from unstimulated bovine lymphocytes show weak labelling of intranuclear granules (x 2800).

All samples below were from bovine lymphocytes 36h ps with con A.

d-f. I1 labels internal granules in Triton nuclei, although the intensity of staining is less than observed *in situ*.

g-i. I1 stains the interior of DNase nuclei in a non-granular, uniform manner.

j-l. Residual nucleoli (arrow) are stained in LMB nuclei.

m-o. M HSB nuclei. Residual nucleoli are stained.

p-r. 2M HSB nuclei. Residual nucleoli (arrows) are stained in some nuclei.

DNase-treated nuclear matrices from bovine lymphocytes did not label with Hoechst or antibody I1.

d

j

m

p

Figure 35. Immunofluorescent labelling of isolated nuclear fractions from bovine lymphocytes by antibodies PI1 or PI2. Phase contrast micrographs (column a-p), Hoechst staining of DNA (column b-q) and immunofluorescence with PI1 (c,f,i) or PI2 (l,o,r); all micrographs x 2500.

a-c. Triton nuclei from unstimulated bovine lymphocytes show weak internal PI1 label as observed for nuclei *in situ*.

d-f. Triton nuclei from bovine lymphocytes 36h ps show strong PI1 labelling of peripheral and internal granules. A type 1 nucleus (at right) is weakly stained.

g-i. Shrunken DNase matrices from bovine lymphocytes 36h ps show similar staining as Triton nuclei using PI1.

j-l. Triton nuclei from unstimulated bovine lymphocytes show primarily peripheral staining using PI2.

m-o. Triton nuclei from 36h ps bovine lymphocytes. PI2 weakly labels internal granules but strongly stains the periphery.

p-r. DNase-treated matrices from bovine lymphocytes 36h ps. PI2 stains the matrix periphery strongly, with few internal granules stained.

a

d

g

m

p

(a) Antibody P1

Extraction of cultured lymphocytes in Triton resulted in only a slight change in the pattern of staining with P1 (fig. 36b,f). The nuclear periphery was stained, although the antigen was located in granules and not in a smooth layer. The intensity of fluorescence was slightly less than in intact cells suggesting some removal of antigen by the nonionic detergent. Digestion of the Triton cells with DNase 1 caused no change in P1 staining (fig. 36c,g), resulting in a pattern comparable to that in isolated DNase nuclei. Extraction of DNase cells with LMB resulted in a considerable loss of staining intensity. The remaining P1 antigen was found in large irregularly spaced granules around the nuclear periphery, interspersed with large unstained areas of the nuclear envelope (fig. 36d,h). This result differs from that obtained using isolated nuclei where there was movement of antigen to the nuclear interior in addition to its extraction. Further extraction of cells with high concentrations of salt (M HSB) removed residual P1 staining. As shown in figure 36 (i,m) the slight amount of P1 staining which remained appeared to stain material which was also stained by Hoechst. Cells extracted by 2M HSB and in situ matrices prepared using DNase ($\pm$ RNase) had no P1 staining (Table 7). These results differ from those with isolated nuclei and matrices where weakly stained intranuclear patches of

PI staining were detected after 2M HSB and DNase. In addition nuclear matrices prepared *in situ* from PtK and 3T3 cells (fig. 39) were stained the same as isolated lymphocyte matrices.

(b) Antibody I1

Triton-extracted cells stained with antibody I1 the same as intact cells (fig. 36) except that there was reduced staining intensity and fewer intranuclear granules per nucleus. DNase digestion of cells abolished all I1 staining; only background fluorescence was observed (fig. 36p). All subsequent samples (LMB cells to DNase-RNase *in situ* matrix) did not stain with I1. Again these results differ from those with isolated nuclei or 3T3/PtK *in situ* (fig. 39). The I1 antigen was removed earlier from *in situ* nuclei without any residual nucleolar staining.

(c) Antibody PI1

The distributions of the antigen recognized by PI1 were only marginally altered during the isolation of *in situ* nuclear matrices from lymphocytes (Table 7). Triton cells stained for PI1 had a typical nuclear staining pattern consisting of internal and peripheral granules (compare fig. 37a,b). DNase-digested cells had the same staining pattern and intensity as intact cells (fig. 37c) although *in situ* DNase nuclei were slightly smaller.

Figure 37d shows that there was a slight loss of PI1 staining intensity in LMB cells and some loss of intranuclear speckles in these nuclei. The nuclear periphery had a uniform distribution of stained granules. The intensity of PI1 staining was slightly greater after HMB extraction, probably due to nuclear shrinkage, but the staining pattern was unchanged (fig. 37i). The results from 2M HSB cells and *in situ* nuclear matrices (fig. 37j-l) were indistinguishable from those in M HSB cells or PtK/3T3 matrices *in situ* (fig. 39).

(d) Antibody PI2

In this experiment bovine cells stained with PI2 had very few internal granules (fig. 38a). Triton cells were similarly labelled by antibody PI2 with slightly lower intensity (fig. 38b). No change was detected after treatment of the cells with DNase 1 (fig. 38c). Figure 38d illustrates the presence of a few intranuclear speckles in nuclei of LMB cells. This observation was consistent with that of Chaly *et al* (1985) using 3T3 cells. All subsequent samples had PI2 staining patterns almost indistinguishable from DNase nuclei, although with slightly lower intensity (fig. 38j-l). The results with *in situ* nuclei also correlate with observations of isolated lymphocyte nuclei and matrices and PtK/3T3 matrices *in situ* (fig. 39), in which PI2 antigen distri-

butions were not altered by the extractions performed.

Figure 36. Immunofluorescent labelling of extracted 36h ps bovine lymphocytes by P1 (a-d,i) and I1 (j-l). DNA was stained using Hoechst (e-h,m and n-p, respectively). P1 staining of (a,e) intact cells, (b,f) Triton cells, (c,g) DNase 1 cells, (d,h) LMB cells and (i,m) M HSB cells. I1 staining of (j,n) intact cells, (k,o) Triton cells and (l,p) DNase 1 cells. All micrographs x 2500.

Figure 37. Immunofluorescent labelling of extracted 36h ps bovine lymphocytes by PI1. PI1 immunofluorescence (a-d, i-l) and Hoechst labelling of DNA (e-h, m-p). PI1 staining of:

- (a,e) Intact cells
 - (b,f) Triton cells
 - (c,g) DNase 1 cells
 - (d,h) LMB cells
 - (i,m) ~~M~~ HSB cells
 - (j,n) 2M HSB cells
 - (k,o) *In situ* DNase matrix
 - (l,p) *In situ* DNase-RNase matrix
- (All micrographs x 2500).

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m		e	p
u			
c			

g.

Figure 38. Immunofluorescent labelling of extracted 36h ps bovine lymphocytes by PI2. PI2 immunofluorescence (a-d, i-l) and Hoechst labelling of DNA (e-h, m-p). PI2 staining of:

- (a,e) Intact cells
 - (b,f) Triton cells
 - (c,g) DNase 1 cells
 - (d,h) LMB cells
 - (i,m) M HSB cells
 - (j,n) 2M HSB cells
 - (k,o) In situ DNase matrix
 - (l,p) In situ DNase-RNase matrix
- (All micrographs x 2500).

Figure 39. Immunofluorescent labelling of *in situ* nuclear matrices in 3T3 and PtK cells by monoclonal antibodies.

P1 stains a fibrogranular intranuclear reticulum in 3T3 (a,e) and PtK (i,m). I1 stains residual nucleoli in 3T3 (b,f) and PTK (j,n). PI1 stains intranuclear and peripheral nuclear matrix granules in 3T3 (c,g) and PtK (k,o). Both 3T3 (d,h) and PtK (l,p) are similarly stained by PI2. (x 1200).

TABLE 7 - Immunostaining of Bovine Lymphocyte Nuclei and Matrices with Monoclonal Antibodies

Nuclear Type		Antibody			
		P1	I1	PI1	PI2
Isolated		P++ I+	I+	P+ I++	P+ I+
Nuclei	1	smooth	granular	granular	granular
Isolated		I+	- *	P+ I++	P+ I
Matrix	1	granular		granular	granular
In situ		-	-	P+ I+	P+
Matrix	1			granular	granular
Isolated		P+++	I++	P++ I++	P++ I+
Nuclei	3	smooth	granular	granular	granular
Isolated		I++	- *	P++ I++	P++ I+
Matrix	3	granular		granular	granular
In situ		-	-	P++ I++	P++
Matrix	3			granular	granular

Nuclear morphotypes assessed by phase contrast microscopy and by costaining for DNA with Hoechst 33258.

P = peripheral; I = internal; "-" = no staining; Number of "+" indicates relative staining intensity.

* - Matrices prepared with DNase 1 showed residual nucleolar stain which was abolished by treatment with RNase A.

DISCUSSION

In order to study nuclear structure and functions many investigators have used immunological probes. Antisera against a number of nuclear components have been used; for example, histones (Mura *et al.*, 1982), DNA (Nymah *et al.*, 1984), RNP (Reuter *et al.*, 1985; Leser *et al.*, 1984), nucleoli (Ochs *et al.*, 1983), lamins (Gerace and Blobel, 1981) or specific chromosomal structures such as centromeres (Moroi *et al.*, 1981). Many of these antibodies are found in autoimmune sera or are of undetermined chemical specificity.

The nuclear matrix is defined as the insoluble residual structure remaining after extraction of isolated nuclei with nonionic detergent, low and high ionic strength and nucleases (Berezney and Coffey, 1977). It is a fraction of considerable compositional complexity (see Chapter 1) to which a number of nuclear functions have been ascribed (Berezney, 1984; Literature Review). The identities and spatial distributions of most nuclear matrix proteins are unknown. When these studies were initiated there were virtually no reports of antibodies specific for nuclear matrix proteins. For this reason mouse hybridomas were produced to obtain specific antisera against components of lymphocyte nuclear matrices. Interestingly a majority of antibody producing hybridomas secreted immunoglobulins which recognized cytoplasmic

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filaments, particularly when bovine lymphocyte nuclear matrix protein was used as immunogen. This is consistent with the ultrastructural appearance of these residual nuclei which were associated with extensive cytoskeletal elements. Two-dimensional PAGE has also shown the presence of actin and vimentin in these matrix preparations (see Chapter 1). There were two types of nuclear antigens recognized by monoclonal antibodies in these studies. One group (P1 and I1) was extracted and/or redistributed during nuclear matrix isolation while another group of antigens (recognized by PI1 and PI2) was not visibly rearranged during isolation of matrix.

A. Antibody P1

Antibody P1 recognized a nonhistone protein of MW 27-30k in 1-D blots (histone H1 migrated at 32-35k in the gels used; Ch. 1). In 2-D gels only acidic to neutral charged proteins are found at 26-30k and these may be P1 antigen. The *in situ* distribution of P1 in interphase is similar but not identical to that of lamins. The smooth perinuclear localization suggests that the antigen may be a lamina or nuclear envelope component. At least some of the P1 antigen may be in a nuclear membrane since extraction of isolated nuclei with nonionic detergent causes movement and/or removal of some of the protein. When intact cells are extracted with Triton X-100 and

DNase 1 the antigen recognized by P1 does not move. In this instance residual cytoskeleton may stabilize the nuclear membrane since it appears relatively intact by EM (Ch. 1). Low and high salt buffers extract a significant fraction of P1 antigen. The remainder may be artifactually entrapped within the intranuclear meshwork. This residual P1 stain is reduced by including both DNase and RNase near the beginning of the procedure. Triton nuclei from unstimulated lymphocytes often showed some internal P1 staining. The anomalous staining of these nuclei could have resulted from release of high levels of proteases and/or nucleases from homogenized con A lymphocytes since Triton nuclei from stimulated lymphocyte preparations (including type 1) show normal peripheral staining.

The data available would appear to suggest that P1 antigen is chromatin-associated. Its extraction from nuclei coincides with chromatin disruption and removal. During mitosis P1 antigen is intimately associated with condensed chromosomes and during con A-induced stimulation lymphoblast nuclei become progressively brighter after staining. The latter observation may be consistent with the massive rearrangement of heterochromatin within lymphoblast nuclei and a requirement for increased numbers of DNA loop attachment sites, some of which may be at the nuclear envelope (Hancock and Hughes, 1982; Murray and

Davies, 1979). Immunofluorescent staining results indicate that P1 antigen may be involved in chromatin organization throughout the cell cycle. During interphase lamins are tightly associated with the residual nuclear envelope and resist extraction during matrix isolation. Thus they may be skeletal lamina proteins (Gerace and Blobel, 1981). On the other hand, proteins such as P1 may be lamina-associated and serve functional roles. In mitosis, as the nuclear lamina disappears, P1 becomes perichromosomal and stains the surface of the chromosome mass. From the increased intensity of P1 staining in prophase it can be inferred that there is far more antigen present at the time of chromosome condensation. P1 could act to constrain condensed chromosome masses throughout mitosis. Furthermore it could act in addition to the chromosome scaffold (Earnshaw and Laemmli, 1983) to maintain chromosome integrity throughout mitosis. Whereas the P1-containing structure which surrounds chromosomes is derived from the nuclear periphery the chromosome scaffold appears to be derived from the intranuclear matrix (Bekers et al, 1981; Earnshaw et al, 1985; Earnshaw and Hack, 1985; Berrios et al, 1985). P1 remains perichromosomal throughout mitosis and is present around the reforming telophase nucleus before lamins are reincorporated into the lamina. Thus P1 could aid reformation of the nuclear envelope by organizing the incorporation of previously

cytoplasmic proteins into an orderly nuclear lamina. In addition, P1 could play a role in anchoring chromatin to the nuclear lamina.

An antigen similar to P1 was detected in a human autoimmune serum by McKeon *et al.* (1984), who named the protein perichromin. Although similar in terms of extraction properties a number of slight differences exist between the two antisera. For example, the staining by P1 is smoother and more uniform in interphase and mitosis. In addition, perichromin is slightly larger (see Table 8). P1 staining of interphase nuclei is sharper and more clearly located at the nuclear periphery. Staining of mitotic chromosomes by P1 and perichromin are similar but not identical. Perichromin stains the peripheries of individual chromatids or chromosome arms whereas P1 stain often rims the entire chromosome mass. P1 and perichromin may represent a class of lamina proteins which are involved in chromatin-lamina interactions.

B. Antibody I1

Antigen I1 is similar to P1 antigen in that it is also extracted from nuclei during matrix preparation. The MW of this molecule was not determined since I1 did not detect polypeptides in nitrocellulose blots or in a solid-phase ELISA. The reasons for this are not apparent although the epitope may have been very sensitive to SDS.

denaturation. Alternatively the antigen may have been very protease-sensitive despite all precautions. A third possibility is that the I1 epitope may not have been a polypeptide but rather a carbohydrate or nucleic acid.

Immunofluorescence of I1 *in situ* showed an intranuclear non-heterochromatin, non-nucleolar distribution in interphase. This staining pattern was dissimilar to that with anti-histone, which was more uniform and less granular (N. Chaly, personal communication). In addition, the pattern was unlike that obtained with antibodies to hnRNP core proteins (Leser *et al.*, 1984) or snRNP proteins (Reuter *et al.*, 1985). Interestingly, the I1 staining pattern resembled that of DNA pol α in interphase HeLa cells or PHA-stimulated human lymphocytes. However, unlike I1, DNA pol α becomes cytoplasmic during mitosis (Nakamura *et al.*, 1984). The *in situ* distribution of I1 implied a possible role in transcription or in modulating chromatin organization.

Extraction of I1 from nuclei during matrix preparation was consistent with chromatin association. DNase 1 treatment changed staining from granular to almost uniform and smooth while low ionic strength buffer extracted nearly all intranuclear I1. Furthermore, during mitosis chromosomes were stained throughout with a granular texture, the I1 stain closely following the Hoechst (DNA) pattern. A transcriptional role for I1

antigen cannot be excluded at present. The persistence of I1 staining of chromosomes during mitosis indicates I1 antigen is not a RNP component since RNP-containing structures are dispersed to the cytoplasm at that time (Leser *et al.*, 1984; Reuter *et al.*, 1985; Clevenger and Epstein, 1984). This study and previous reports have shown that nuclear proteins can be arbitrarily classified according to their mitotic distributions into one of three categories (see Table 3). Some proteins remain with condensed chromosomes throughout mitosis due to known functional binding to DNA or chromatin. These include histones, DNA-binding proteins (Woodcock *et al.*, 1984) and chromosome scaffold proteins (eg. topoisomerase II; Earnshaw *et al.*, 1985). A second group of proteins is chromosome-associated during mitosis but not visibly during interphase and includes P1, I1, perichromin (McKeon *et al.*, 1984), and nucleolar B23 (Ochs *et al.*, 1983). The bulk of nuclear NHP are dispersed in the cytoplasm in mitosis and are often associated with particulate components. This fraction includes lamins (Gerace and Blobel, 1981), snRNPs (Reuter *et al.*, 1985), hnRNP core proteins (Leser *et al.*, 1984), interchromatin granules (Clevenger and Epstein, 1984), DNA pol α (Nakamura *et al.*, 1984), PI1 and PI2.

During lymphocyte stimulation by con A larger nuclei were progressively brighter after I1 staining. It is not

apparent whether this was due to increased concentrations of I1 antigen and/or simply to nuclear enlargement and maintenance of I1 concentration. Some caution is required in attempting to correlate immunofluorescent staining intensity with protein levels, particularly when there are subtle changes in brightness. It is possible that as condensed chromatin in lymphocytes disaggregates some epitopes are unmasked. Use of synchronized cells and pulse-chase experiments are required to accurately determine cell cycle-dependent changes in protein concentration. On a subjective level it is possible to infer that the concentration of I1 antigen per nucleus appears to increase following con A stimulation. This increase could be associated with unfolding or disaggregation of chromatin in types 2 and 3 nuclei and the requirement for more intranuclear chromatin anchoring sites.

Immunofluorescent data have shown that the antigens recognized by P1 and I1 are highly conserved in animals, plants and fungi. These results suggest that I1 and P1 may be important chromatin-associated proteins involved in organization of chromosomes throughout the cell cycle.

An important result derived from the present studies is that presence of a protein within the final nuclear matrix fraction is not always structurally meaningful. The antigens recognized by P1 and I1 are largely extracted

from nuclei during isolation of the nuclear matrix and the remaining molecules are extensively redistributed within the structures. The presence of P1 and I1 within the original immunogen was probably due to this incomplete removal from the DNase matrices used to immunize mice. RNase treatment reduces the levels of I1 and P1 antigens in final matrices (Chaly et al, 1985). I1 antigen appears to adhere artifactually to residual nucleoli whereas P1 antigen could associate with an intranuclear RNP- derived network. These results may be significant in view of the numerous functions ascribed to the nuclear matrix from strictly biochemical approaches in which functional elements show matrix association (for review see Berezney, 1984). In order to more precisely localize nuclear functions it will be necessary to show that active molecules which copurify with matrix are ultrastructurally undisturbed by fractionation treatments (eg. Smith et al, 1984). Despite their extraction from nuclei and rearrangements *in vitro* it remains plausible that P1 and I1 antigens have important functions in chromatin- nuclear matrix associations *in vivo*. To discover these functions it will be necessary to experimentally manipulate more intact cell fractions and/or isolate the antigens and determine their biochemical properties and affinities (eg. binding nucleic acids).

C. Antibodies PI1 and PI2

Unlike P1 and I1, antibodies PI1 and PI2 recognize antigens which are not extracted or significantly redistributed during matrix preparation in situ or in solution. Both antigens are found in fibrogranular networks at the nuclear surface and in the IM. Some loss of PI2 internal staining components may be due to the protease sensitivity of the intranuclear matrix. It is significant that the patterns of PI1 and PI2 staining are the same regardless of whether matrices are isolated in the presence or absence of RNase. Bouvier et al (1985b) have suggested that the intranuclear matrix is an artifact derived from RNP networks.

During mitosis PI1 and PI2 are dispersed in the cytoplasm like several other nuclear NHP (eg. hnRNP, snRNP, lamins). Nuclear stain reappears in telophase as nuclei reform. Both antigens are moderately conserved as nuclei from all mammalian cells tested gave similar staining. Neither PI1 nor PI2 is as highly conserved as P1 and I1 and some species- and/or tissue- specific staining variations were detected.

PI1 labelled a 60k polypeptide on nitrocellulose blots of nuclear or matrix proteins. Based on immunofluorescence of interphase cells the antigen was not a lamin but was one of the numerous weak 40-80k bands visible in gels of nuclear matrix proteins. PI1 antigen

may be one of the series of basic proteins resolved in 2-D gels which are presumed to be components of the IM (see Chapter 1 Discussion). The novel interphase immunofluorescent staining pattern did not resemble staining of lamins, hnRNP, snRNP or histones (N. Chaly, personal communication).

Different nuclear distributions of PI1 antigen are found between type 1 and types 2 and 3 cells. Whereas PI1 stains primarily intranuclear granules in type 1 nuclei, the larger nuclei also have bright uniformly distributed peripheral granules. This increased level of PI1 and the different in situ distribution could be related to the structural complexity observed in type 2 and 3 nuclei. Possible functions for PI1 based on staining results and putative matrix roles in vitro could include transcription or RNP transport (nucleocytoplasmic exchange). PI1 may be involved in preparation of cells in G1 phase for DNA replication. In cycling cells (eg. 3T3) this may account for the variable staining patterns seen. Such a hypothesis would also explain the changes in PI1 staining during con A stimulation of lymphocytes. PI1 could also conceivably be a structural component of a highly ramified fine nuclear skeleton, as observed by Brasch (1982) in situ and isolated by Agguter (1985). However, none of the antibodies studied here stain the entire nuclear interior diffusely as would be expected for such a protein.

However, PI1 (and PI2) may be involved in transient intranuclear and perinuclear binding of chromatin or RNP to such a nuclear skeleton.

Antibody PI2 recognizes at least 3 polypeptides in nitrocellulose blots (ie. 130, 70 and 35k). The smaller molecules may be degradation products of p130 or subunits of the larger protein. The possibility that these subunits are cross-linked by disulphide bridges in NaTT was not determined. Proteins sorted in SDS-PAGE gels were reduced in 2-mercaptoethanol to break disulphide linkages prior to electrophoresis. Incomplete reduction of disulphide bridges between polypeptides could account for the observed MWs of PI2 antigens being multiples of 35k (ie. monomers, dimers and tetramers).

As observed with PI1 the staining pattern of PI2 is unlike those of other nuclear proteins (eg. hnRNP core, snRNP, histones). In most cell types a variable number of peripheral and intranuclear granules are stained although mouse lymphocyte nuclei only stain peripherally. Larger lymphocyte nuclei seem to contain greater amounts of PI2 antigen although the difference between nuclear types is less obvious than with PI1. The appearance of greater amounts of PI2 during lymphocyte stimulation could have structural or functional implications. Its primarily peripheral localization might indicate a role for PI2 in nucleocytoplasmic exchange. As suggested for PI1 antigen

above, the antigen recognized by PI2 may link other molecules to the nuclear matrix and/or have enzymic properties. The chromosome scaffold/nuclear matrix protein Sc1/topoisomerase 2 is postulated to have such a dual role in chromosomes (Earnshaw et al, 1985; Earnshaw and Heck, 1985).

The antigens recognized by PI1 and PI2 are novel IMP which have unique intranuclear distributions. Although functional roles have not been established they represent nuclear matrix proteins which resist extraction from nuclei after a variety of treatments without undergoing redistribution. In this regard they are similar to the lamins (Gerace and Blobel, 1981) but they represent a class of proteins which exist in a genuine intranuclear network which remains intact during nuclear matrix isolation. In this regard they provide further evidence that the IM in nuclear matrix is a real structure, not simply a random precipitation of nucleoplasm.

TABLE 8 - Distributions of Some Nuclear Antigens
During the Cell Cycle

Antigen	MW(kd)	Interphase	Mitosis	Ref. *
Histones	14-18, 32-35	Uniform, fine granules	uniform c'somal	15
AhnA1 (DNA- binding)	55	"	C'somal	2
Centromere	?	Large internal speckles	Centromere	3,4
Sc1, Sc2	170, 135	Internal granules	C'some core	5,6
P1	27-30	Smooth, peripheral	Peri- c'somal	1,17
I1	?	Internal granules	C'somal granules	1,17
Perichromin	33	Peripheral	Peric'somal	7
Nucleolar B23	37	Nucleolus	C'some- associated	14

Nucleolar C23	110,68	"	" (NOR)	14
Lamins	60-72	Smooth, peripheral	Cytoplasm	8
snRNP	11-32,69	Patchy, internal granules	"	9
hnRNP core	32-44	"	"	10
IG	41,105	Internal granules	"	11
DNA pol a	140-150	"	"	16
ANP	30	"	"	12
PI1	60	" & peripheral granules	"	1,17
PI2	130,70,35	"	"	1,17
MSA	?	Internal speckles	Spindle	13

Table 8 (continued)

*C'somal = chromosomal; IG = interchromatin granules; ANP = acidic nuclear protein; MSA = mitotic spindle apparatus; NOR = Nucleolus organizing region.

1. Bladoh et al(1983); 2. Woodcock et al(1984); 3,4. Moroi et al(1980,1981); 5. Earnshaw et al(1985); 6. Berríos et al(1985); 7. McKeon et al(1984); 8. Gerace and Blobel(1981); 9. Reuter et al(1985); 10. Leser et al(1984); 11. Clevenger and Epstein (1984); 12. Krohne and Franke (1980); 13. McCarty et al(1981); 14. Ochs et al(1983); 15. Mura et al(1982); 16. Nakamura et al (1984); 17. Chaly et al (1984).

SUMMARY AND CONCLUSIONS

From earlier studies using mouse and human lymphocytes it was shown that nuclei in con A-stimulated lymphocytes undergo significant structural reorganization as cells proceed from G0 through G1 and S phases of the cell cycle. Condensed chromatin disaggregates and an elaborate interchromatinic network forms. Preliminary analyses indicated that nuclear matrices from such cells were markedly different from those in quiescent lymphocytes (Setterfield et al, 1983a, 1985). It was confirmed here that bovine lymphocytes respond similarly to concanavalin A as mouse and human lymphocytes.

In this study it was shown that residual structures from isolated bovine lymphocyte nuclei ($\pm$ con A) resisted extraction by nonionic detergent, low and high ionic strength and nucleases. Some ultrastructural changes were noted as chromatin was extracted by LMB and HSB buffers. In general the morphologies of isolated residual nuclei resembled the *in situ* interchromatinic material (IM) and lamina. Differences in nuclear ultrastructure and volume between inactive and active nuclei were maintained in residual nuclear fractions and nuclear matrices (NM). Matrices from unstimulated bovine lymphocytes consisted of a peripheral lamina and minimal IM, whereas NM from stimulated cells had a lamina, residual nucleoli, nuclear

bodies (NB) and an extensive IM. The latter was composed of salt- and nuclease-resistant 2-6 nm fibrils and 10-25 nm granules.

Compaction of residual nuclei and NM may have resulted from inclusion of divalent cations in buffers, intermolecular disulphide bridges and/or chromatin extraction. This reorganization indicates that caution is required when ascribing functional roles to NM from gross biochemical analyses. Extracted nuclei within unhomogenized cells were not compacted or extensively reorganized, implying a possible contribution by the cytoskeleton in maintaining an open nuclear morphology. A tight association between isolated NM and residual cytoplasmic filaments (presumably intermediate or microfilaments) was shown. Vimentin and actin were identified as major components of lymphocyte NM by 2-D PAGE, particularly in NM from unstimulated cells.

Residual NB were found to be components of large nuclear matrices. Their ultrastructure was unaffected by extraction of nuclei and tight connections to a residual fibrogranular IM were salt- and nuclease-resistant. Possible roles for NB in matrices were suggested.

One-dimensional SDS-PAGE showed that primarily histone protein was extracted from nuclei during isolation of NM. Several nonhistone proteins (NHP) were thereby enriched in residual nuclear fractions, including those of

MW 42-85k, 115, 130 and 170k. However, few marked composition differences between nuclear matrices from unstimulated vs con A stimulated Bovine lymphocytes were observed. On the other hand, 2-D PAGE resolved a large number of NHP in matrix preparations. In general NM from unstimulated lymphocytes had relatively simple polypeptide contents, with acidic to neutral species most abundant. Lamin B, actin and vimentin were major components of these nuclear matrices. This composition was consistent with the simple appearance of *in situ* nuclei and isolated NM from quiescent cells. Nuclear matrices from lymphocytes 18h ps contained largely the same proteins but with several additional polypeptides. There was an abundance of neutral to basic charged proteins (MW 32-85k), including numerous hnRNP proteins. Lamins A and C appeared as minor proteins and an acidic 38k polypeptide increased significantly in amount by 18h ps. In blast nuclei (36h ps) the changes in NM protein content (and structure) were even more pronounced. Increased complexity of 2-D protein gels appeared to be due to de novo synthesis of novel NHP and post-translational modification of NM proteins. Increased numbers of NHP in nuclear matrices coincided with increased nuclear volume and structural elaboration and elevated levels of transcription and RNA processing (Setterfield et al, 1983a and this study).

This study differed from earlier ones where no significant differences in protein composition were observed by 1-D PAGE between active and inactive tissues or cells (see Literature Review). Lectin-stimulated lymphocytes provided a suitable model system for studying metabolic and structural changes during cell activation. Furthermore, two-dimensional gel analyses were clearly necessary to characterize the compositionally complex NM; 1-D gels were of limited value.

The nuclear matrix was structurally similar to an RNP-rich *in situ* IM and several presumed transcription-associated NHP were in NM. Thus the matrix may act to spatially order the transcriptional and RNP transport apparatus. An involvement in ordering DNA replication or chromatin structure cannot be excluded. The nuclear matrix probably consists of structures or molecules attached to a fine fibrillar underlying skeletal structure as observed *in situ* (Brasch, 1982; Setterfield *et al.*, 1985).

Monoclonal antibodies were raised against bovine (P1 and I1) and mouse (PI1 and PI2) lymphocyte nuclear matrix proteins. Antigen P1 (27-30k) was located in the nuclear periphery; I1 was in the nuclear interior while PI1 (60k) and PI2 (34k, 70k, 130k) were in both compartments. The levels of all 4 antigens increased in lymphocyte nuclei

after con A stimulation, as determined by immunofluorescence.

Antigens P1 and I1 were chromosome-associated throughout mitosis and were redistributed or removed from interphase nuclei as chromatin was extracted during NM isolation. This suggested they might be involved in modulating chromatin organization throughout the cell cycle. P1 and/or I1 antigens could mediate chromatin-NM interactions during interphase. Their conservation in evolution implied important roles in nuclei for P1 and I1 antigens. The redistribution of these antigens during NM isolation showed that, although largely undetected, nuclease and salt extraction of nuclei can seriously disrupt *in vivo* associations between some nuclear components (see also Mirkovitch et al, 1984).

PI1 and PI2 were conserved in evolution, although less than P1 or I1, and had novel interphase distributions. They were not visibly rearranged within residual nuclear structures during NM isolation. During mitosis PI1 and PI2 antigens became cytoplasmic like several other NHP. From available data it was suggested that PI1 and PI2 may be associated with transcription or processing and transport of RNP and could conceivably mediate binding of (nucleo)proteins to a nuclear skeleton. The antigens recognized by PI1 and PI2 existed in a genuine intranuclear fibrogranular network which survived

nuclear matrix isolation indicating that, although some matrix-associated proteins are labile, the arrangement of lamina and IM within the isolated nuclear matrix seems real and not due to random precipitation of nuclear material.

APPENDIX 1 - ISOELECTRIC FOCUSING (IEF) GELS *

1. For 5 gels (19cm x 3.2mm) use:

5.66g ultrapure urea (Schwarz-Mann)

1.0 ml 28.8% acrylamide/1.62% bis
(17.8:1; Sigma)

2.0 ml 10% (w/v) Nonidet P-40

2.3 ml distilled water

with the following additions,

EQUILIBRIUMNONEQUILIBRIUMAmpholytes (Bio-Rad)

100 ul	3-10	600 ul
300 ul	5-7	---
200 ul	6-8	---

Catalysts

15 ul	Ammonium Persulphate	20 ul
	(100mg/ml; Sigma)	

8 ul	Riboflavin (0.5mg/ml)	14 ul
	(Bio-Rad)	

5 ul	TEMED (Bio-Rad)	5 ul
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Tray Buffers

bottom	A. 10mM H ₃ PO ₄	top
top	B. 40mM NaOH, 20mM Ca(OH) ₂	bottom

Electrodes

bottom

Positive

top

top

Negative

bottom

2. Gels polymerized under UV 4 hours to overnight.

3. Electrophoresis conditions:

Equilibrium - 0.5mA per gel constant current,
increasing to 800V (17h).

Hyperfocus 1h at 1000V.

Nonequilibrium - 11h at 400V (constant).

(NEPHGE)

* Peters and Comings (1980).

APPENDIX 2 - SECOND DIMENSION SDS-PAGE GELS *

1. Stacking gel (25 ml):

5% (w/v) acrylamide/bis (36.5:1; Sigma)

2mM EDTA

0.1% (w/v) high purity SDS (Bio-Rad)

125mM Tris-HCl (Sigma), pH 8.8

Catalysts: 125ul 10% (w/v) ammonium
persulphate (Sigma)

25 ul TEMED (Bio-Rad)

Polymerized at least one hour.

2. Running gel (260 ml):

10% (w/v) acrylamide/bis (36.5:1; Sigma).

2mM EDTA

0.1% (w/v) high purity SDS (Bio-Rad)

375mM Tris-HCl (Sigma), pH 6.8

Catalysts: 2.6ml 10% (w/v) ammonium
persulphate (Sigma)

145ul TEMED (Bio-Rad)

Polymerized overnight.

3. Tray Buffer: 192mM glycine (Bio-Rad), 25mM Trizma
(Sigma); 0.1% (w/v) SDS (Bio-Rad); 2mM EDTA

* Peters and Comings (1980)

APPENDIX 3 - SILVER STAINING OF 2-D GELS *

Gels which had been stained with Coomassie were treated sequentially at room temperature with the following solutions:

1. Fix in 50% (v/v) ethanol, 10% (v/v) acetic acid;
2 x 2h.
2. Wash in 25% (v/v) ethanol, 10% (v/v) acetic acid;
2 x 1h.
3. Wash in 10% (v/v) ethanol, 0.5% (v/v) acetic acid;
2 x 1h.
4. Equilibrate in 0.19% (w/v) silver nitrate at least 2h.
5. Rinse with distilled water 10-20 sec.
6. Reduce in 0.3% (w/v) formaldehyde, 0.09% (w/v) NaBH_4
in 0.75N NaOH until first spots appear in gel.
7. Enhancement in 0.75% (w/v) Na_2CO_3 ; photograph
in this solution and store.

* Adapted from Sammons et al (1981).

APPENDIX 4 - STANDARDS USED IN 2-D PAGE

Standard	Notation	pI	MW
1. 3-Phosphoglycerate kinase	P	8.1	43.5k
2. Phosphorylase a	Pa	7.8	94k
3. Carbonic anhydrase	C	7.4	29k
4. Bovine serum albumin	B	6.7	65k
5. Actin	A	6.1	43.5k
6. Soybean trypsin inhibitor	S	5.5	21.5k
7. Internal std. (hnRNP core)	Hn	>8.4	36k

From Comings and Peters (1980).

APPENDIX 5 - HY CULTURE MEDIUM FOR HYBRIDOMAS

HY medium used for culture of mouse hybridoma cells was prepared according to Kennett (1980) and consisted of Dulbecco's modified Eagle's medium containing:

- high glucose content
- 10% NCTC 109 medium
- 20% heat-inactivated foetal calf serum
- 0.15mg/ml oxaloacetate
- 0.05 mg/ml pyruvate
- 0.2 U/ml insulin
- 100 U/ml penicillin
- 100 ug/ml streptomycin

For HAT selection of hybridoma cell lines the following ingredients were added to HY medium before the fusion products were grown

in 96-well culture plates:

- 0.1mM Hypoxanthine
- 0.4uM Aminopterin
- 16 uM Thymidine

After 6 days the culture medium was replaced by HY medium containing thymidine and hypoxanthine but lacking aminopterin.

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